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Carbon Mineralization by Mixed Cultures

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1. INTRODUCTION

Carbon mineralization is defined strictly as the decomposition of carbon-containing organic matter to produce carbon dioxide or methane. Thus, it parallels biogeochemical processes in the nitrogen and sulphur cycles where ammonia and hydrogen sulphide are evolved, respectively. This chapter adopts a rather narrower view of mineralization and concentrates upon the predominantly extracellular events which occur during the saprophytic degradation of carbohydrate polymers and associated carbon-containing structures. Thus, the absorption and intracellular catabolism of relatively low molecular weight carbon compounds will not be considered in any detail nor will the enzymic activities of pathogenic microorganisms and the autolytic reactions concerned with microbial morphogenesis.

Nevertheless, any self-imposed constraint based upon the location of enzyme-substrate interactions or the physiological role of the enzyme

must be somewhat flexible for a number of reasons. For example, microorganisms have variable capabilities in terms of their absorption of substrates either due to cell wall porosity (e.g. the cell walls of Gram-positive bacteria have much larger pores than those of Gram-negative bacteria) or to the presence of specific active transport mechanisms. As a consequence, a potential substrate which is absorbed and metabolized by one species may require additional extracellular transformations before it can be utilized by another microorganism. An appropriate example is that some bacteria may find cellobiose or even glucose oligomers suitable as intracellular substrates; others may require monomeric glucose.

The molecular size and solubility of a substrate are not the only factors determining its ease of absorption. Stereochemical and in particular ionogenic properties are also important and may fluctuate according to the immediate environment. In soils and sediments, the clay and humic components have an extensive unit surface area (up to 800 m^2 (g soil colloid)$^{-1}$) and are predominantly negative charged. As a result organic moieties tend to accumulate at the solid–liquid interface where a host of adsorptive phenomena will influence their availability as substrates and, therefore, their rate of mineralization (Burns, 1979, 1980). As a consequence, the rates and routes of mineralization *in vivo* may bear little relationship to that recorded *in vitro*. This is especially true for ionic substrates—proteins, amino acids and a host of xenobiotic compounds—but is also valid for carbohydrates.

Another factor to consider when defining the boundaries of extracellular mineralization is that many intracellular hydrolytic enzymes become externalized following the death and lysis of their parent cell. For example, urease, a key carbon and nitrogen cycle enzyme, is cytoplasmic in terms of its original functional location, yet will survive and retain its activity for long periods in cell debris or if complexed with soil clay and humic colloids. Thus, urea mineralization (and, therefore, the final stages of purine and pyrimidine breakdown) may occur extracellularly even though the substrate has a low molecular weight and is highly soluble, and despite the presence of large numbers of ureolytic microorganisms.

Finally, one needs to define extracellular, a term which means different things to different microbiologists. Some (Glenn, 1976) have suggested that all catalyses outside the cytoplasm should be regarded as extracellular, including events within the periplasmic space of Gram-negative bacteria and those within the outer wall. Others regard enzymes attached to and projecting from the cell wall as extracellular whilst still others would prefer to restrict the adjective to enzymes which are spatially removed from their cellular origins: that is, have dif-

fused away from their parent cells during normal growth and reproduction (Pollock, 1962). These various interpretations are further confused by such factors as the changing location of many enzymes with time: periplasmic enzymes may leak during growth, cytoplasmic enzymes may escape from lysed cells and at any particular moment many truly extracellular enzymes may be *en route* to the outer membrane and thence to the external environment. In addition, the same enzyme may not have the same location in different species: extracellular enzymes (e.g. penicillinase) in Gram-positive bacteria may be periplasmic in Gram-negative organisms. Indeed free extracellular enzymes are uncommon in Gram-negative species (Lory and Colliers, 1978; Pavlovskis and Wretlind, 1978) and most of the secreted enzymes are found in the periplasmic space or the outer membrane.

For the purposes of this chapter extracellular enzymes are regarded as those which catalyse extracellular events and, therefore, have at least some physical contact with the ambient medium. I shall, however, be less rigid when it is uncertain exactly where a reaction has occured or when an intracellular event is an essential step within a predominantly extracellular sequence.

2. CARBON-CONTAINING SUBSTRATES: ORIGINS AND COMPOSITION

2.1 Introduction

Enormous quantities of organic debris are deposited each year into terrestrial and aquatic environments: plant litter alone accounts for between 1.0 and 15.3 tonnes of organic matter hectare $^{-1}$ annum^{-1} (Williams and Gray, 1974) and the root systems of green plants produce cell debris and soluble organic materials during growth. Estimates of root biomass in the top 30 cm of soil are of the order 440 to 1575 g m^{-2} (Dickinson, 1974). In addition, animals and their excreta and dead microbial cells provide a significant residue that, like the plant fractions, is degraded by the microflora in order to maintain the steady-state condition between carbon assimilation and mineralization. Estimates of soil microbial biomass range from 50 to 3710 μg biomass carbon (g soil)$^{-1}$ (Jenkinson and Ladd, 1981). One estimate of fungal biomass associated with decomposing pine needles is 4 to 5 mg (g plant material)$^{-1}$, a figure equivalent to 3000 to 4000 m mycelium g^{-1} (Berg and Soderstrom, 1979).

A large proportion of the annual input of organic matter is carbon (*ca.* 3.0×10^{10} tonnes—Norkrans, 1967)—often as simple sugars

(hexoses and pentoses), their derivatives (e.g. amino sugars, uronic acids, sugar alcohols, methylated sugars, deoxysugars) or as their appropriate polymers. Carbon also occurs as a component of lignin, urea, alcohols, fatty acids, purine, and pyrimidine bases and lipids together with a host of xenobiotic compounds. Somewhere between 50% and 75% of the dry weight of plant tissues is carbohydrate, while microbial cells contain up to 60% organic carbon (Lieth, 1975). The precise amount of carbon in, as well as its proportional contribution to, a cell, tissue, or entire organism is determined by species and age. For instance, mature plants have a higher percentage of cellulose, hemicellulose, and lignin even though water-soluble carbohydrates may predominate in young plants.

The principal substrates for carbon cycle microorganisms are listed in Table 1 and their origins and chemical structure outlined below.

2.2 Plant Carbon

2.2.1 Cellulose

Cellulose is the most abundant polymeric constituent of plant material. Mature wood contains 40 to 50% cellulose, leaves 10% and cotton 98% and it has been estimated that the annual global production is somewhere in the region of 1×10^{11} tonnes. A significant proportion of this cellulose (*ca.* 5 to 10%) occurs as municipal, industrial, and agricultural waste and it is not surprising that great efforts have been made to understand the physical, chemical, and microbiological factors which influence its decay. Increasingly cellulosic waste is seen as a renewable resource (Gaden *et al.*, 1976; Tsao, 1978; Ghose and Ghosh, 1979) and recent studies have emphasized the potential of the polysaccharide not only as a source of methane and fermentable sugars (Ladisch, 1979), but also as a substrate for microbial biomass or single cell protein production (Flickinger and Tsao, 1978). As a result, polysaccharide microbiology and biochemistry is dominated by cellulose, and it is appropriate that a discussion of carbon mineralization should be centred upon this plentiful substrate.

Cellulose is a linear homopolymer composed of β-(1-4) linked glucose residues. In the case of cotton, as many as 10 000 residues make up the cellulose molecule indicating a molecular weight for native cellulose of $>1.5 \times 10^6$ and a total length in the region of 5 μm. Model cellulose substrates, such as filter paper, have a much lower degree of polymerization (500 to 2000 glucose units) as does the cellulose of mature wood (1500 to 2100 glucose units).

In the plant cell wall individual cellulose molecules associate to form

Table 1. Principal carbon-containing polymers of plant, animal, and microbial organic matter.

Polymer	Predominant Structure (where known) and Component Monomers	Origin
Cellulose	β-(1-4)-D-glucan	Plants, fungi and a few bacteria
Hemicellulose	β-(1-4)-D-xylan xyloglucan arabinogalactan glucomannan arabinoxylan galactoarabinoxylan glucoarabinoxylan	Plants
Pectin	α-(1-4)-D-galacturonan arabinogalactan rhamnogalacturonan xyloglucan	Plants and fungi
Starch (amylose)	α-(1-4)-D-glucan	Plants and fungi
Starch (amylopectin)	α-(1-4)-D-glucan with α-(1-6)-D linked branches	
Inulin	β-(2-1)-D-fructosan	Plants
Fructosan	β-(2-6)-D-fructosan	Plants
Lignin	coniferyl, sinapyl and *p*-coumaryl alcohols	Plants
Glycoprotein	L-arabinose D-galactose D-glucose D-mannose L-rhamnose uronic acids	Animals, plants and microorganisms
Chitin	β-(1-4)-N-acetylglucosamine	Animals (arthropods), fungi and a few protozoa and diatoms
Chitosan	β-(1-4)-glucosamine	Fungi (Zygomycotina)
Glycogen	α-(1-4)-D-glucan α-(1-4)-D-glucan with α-(1-6)-D linked branches	Plants, fungi and animals
Peptidoglycan	N-acetylglycosamine with β-(1-4)-D linked N-acetylmuramic acid	Bacteria

Table 1 (*Continued*)

Polymer	Predominant Structure (where known) and Component Monomers	Origin
Teichoic acid	Glycerol or ribitol phosphate containing sugar, amino sugar and D-alanine substituents	Bacteria (Gram-positive)
Lipopolysaccharides	D-abequose D-glucose D-galactose D-mannose L-rhamnose L-fucose N-acetylglycosamine L-glycero-D-mannoheptose 3-deoxy-D-mannooctulosonate + D-glucosaminyl β-(1-4) or β-(1-6)-D glucosamine	Bacteria (Gram-negative)
Glucans	α-(1-3)-D-glucan α-(1-6)-D-glucan α-(1-3)-D-glucan with α-(1-4) and α-(1-6)-D linked branches	Fungi and bacteria (e.g. *Leuconostoc* spp.)
Mannans	α-(1-6)-D-mannan with α-(1-3)-D and α-(1-2)-D linked branches	Fungi
Dextrans	α-(1-6)-D-glucan	Bacteria (Lactobacteriaceae)
Levans	β-(2-6)-D-fructan	Bacteria (e.g. pseudomonads, *Bacillus* spp.)
Pullulan	α-(1-4)-D and α-(1-6)-D maltotriose	Fungi (*Aureobasidium pullulans*)
Xanthans	β-(1-4)-D-glucan with α-(1-3) linked side chains of mannose and glucuronic acid	Bacteria (*Xanthomonas* spp.)
Alginate	β-(1-4)-D-mannuronic acid and α-(1-4)-L-guluronic acid	Brown algae and a few bacteria

microfibrils which, in turn, aggregate to produce fibrils. The fibrils are stabilized by the formation of hydrogen bonds between the hydroxyl groups of adjacent chains. Some areas of the fibril are tightly bound and are known as ordered or crystalline regions whilst more loosely associated zones are described as amorphous or paracrystalline. This distinction between physically different regions of the cellulose is important when considering the accessibility of the substrate to cellulolytic microorganisms and cellulases. Cellulose structure is discussed in detail by Sihtola and Neimo (1975), Tsao *et al.* (1978), Cowling and Brown (1979), and Fan *et al.* (1980).

2.2.2 Hemicelluloses

Hemicelluloses are a diverse group of alkali soluble polysaccharides structurally associated with cellulose in plant cell walls. Mature wood may contain greater than 30% (w/w) hemicellulose, whilst straw contains about 20% (w/w). Hydrolysis of hemicullulose yields a mixture of hexos sugars (e.g. D-galactose, D-mannose), pentose sugars (D-xylose, L-arabinose) and uronic acids (glucuronic acid, galacturonic acid). Xylose polymers (xylans) may account for 7 to 30% of the plant weight whereas wheat straw hemicellulose is 90% (w/w) xylan. The occurrence and biochemistry of many of the hemicellulose polymers has been reviewed by Dekker and Richards (1976).

2.2.3 Pectin

Pectin substances form a minor component of the cell walls of higher plants rarely contributing more than 5% of the total weight. Pectins are complex polysaccharides composed of galacturonic acid moieties in which the carboxyl groups may be esterified to various degrees with methyl groups. Pectic substances are often divided into pectins, pectinic acids, protopectin, and pectic acids according to their water solubility and the prevalence of methyl ester linkages.

2.2.4 Starch

Starch is a linear or branched glucan serving as a reserve food source for plants and stored in roots (tubers), stems (corms), and swollen leaf structures (bulbs). It is also found in high concentrations in cereal grains (e.g. barley, maize, wheat, oats) and is second only to cellulose as the most common hexose polymer in the plant world. Starch is composed of an essentially linear structure (in which the glucose dimer, maltose, is linked in the α-(1-4) position) as well as of side chains attached through α-(1-6) linkages. The former is known as amylose; the latter as amylopectin.

Agar and alginates

Agar is a complex polysaccharide found in marine red algae and is composed of a neutral agarose fraction and an ionic agaropectin fraction. Both components are linear polymers and thought to consist of alternating units of β-(1-3)-D-galactose and α-(1-4)-3,6-anhydro-L-galactose. The galactoside units of the agaropectin may be methylated or contain pyruvic or sulphuric acid residues (Duckworth and Yaphe, 1971; Izumi, 1972). Porphyran and carrageenan are closely related red algal polysaccharides but which are sulphated to different degrees (Percival and McDowell, 1967).

Alginates are major structural polysaccharides of marine brown seaweeds (e.g. the genera *Laminaria*, *Ascophyllum*, *Macrocystis*) but are also deposited as exopolysaccharides by certain bacteria (e.g. *Azotobacter vinelandii*, *Pseudomonas aeruginosa*). The function of bacterial alginates may be to protect the cell from dehydration (Carlson and Matthews, 1966), to prevent heavy metals from entering the cell (Den Dooren de Jong, 1971) or as a diffusion barrier to oxygen (Postgate, 1974). Alginate synthesis and structure have been discussed recently by Jarman (1979).

Lignin

Lignin is an important carbon-containing constituent of vascular plants and forms some 15 to 35% by weight of wood. Predictably, lignin composition and quantity vary with the age and species of the plant: mature plants have more lignified tissue than young plants; conifers generally contain more lignin than do hardwood species. Lignins are polymers of coniferyl, sinapyl and *p*-coumaryl alcohols joined by a variety of intermonomer linkages. Ether bonds are the dominant linkages but alkyl-alkyl, alkyl-aryl and aryl-aryl also exist.

2.3 Animal Carbon

Animal tissues generally contain a smaller proportion of carbohydrate than do those of plants and microorganisms. Chitin, however, is a major organic component of arthropod exoskeletons and it has been estimated that a single species of crab produces millions of tonnes of chitin per year. The structure of this abundant carbon (and nitrogen) source is discussed on p. 483.

A second important animal carbohydrate is glycogen. This substance is similar in both structure and function to the starch of plant cells. In other words, it is a polymer composed of α-(1-4) and α-(1-6) linked glucose units and acts as a medium for energy storage.

A large number of animal carbohydrates occur as glycoproteins. Glycoproteins are proteins to which carbohydrates are linked through glycosidic bonds. The carbohydrate moiety varies in size from mono- to polysaccharide. Glycoproteins are common constituents of animals but are also found in some plants and microorganisms. They are also extremely diverse in structure and function, for example, virtually all the proteins of human plasma (excepting albumin) are glycoproteins and glycoproteins have structural (collagen, hyaluronic acid, chondroitin sulphates, heparin), protective (immunoglobulins), hormonal (thyroglobulin), and enzymic (acetylcholinesterase) functions. They also serve as food reserves (casein, ovalbumin).

Sugars which are commonly found in glycoproteins include: galactose, mannose, glucose, L-fucose, N-acetyl-glucosamine, N-acetyl-galactosamine, xylose, and L-arabinose. The proportion of sugar varies from 0.5% in some collagens to greater than 80% in blood-group substances of unknown function. Animal carbohydrate is also contained in nucleic acids and glycosides.

2.4 Microbial Carbon

2.4.1 Chitin and chitosan

It is often stated that chitin is second only to cellulose in abundance and it was once estimated that marine copepods alone produce 1×10^9 tonnes of chitin per year (Tracey, 1957) and that cellulose and chitin together account for 1×10^{10} to 1×10^{12} tonnes of the annual output of new carbohydrate. Within the animal kingdom the exoskeletons of arthropods (insects, spiders, crabs, lobsters) may contain up to 80% chitin (Jeuniaux, 1963). Chitin is also an essential structural component of fungi whose cell walls frequently contain greater than 10% by weight of this polymer. A few protozoa and marine diatoms also contain small amounts of chitin. Chitin is a long chain polymer of N-acetylglucosamine linked by β-(1-4) bonds and is thus chemically related to cellulose. Like cellulose, chitin also forms crystalline structures which are arranged side by side and linked through hydrogen bonds. A detailed description of chitin and its properties can be found in Muzzarelli (1977).

Chitosan occurs in the hyphal walls of zygomycete fungi, such as the genera *Mucor, Mortierella*, and *Rhizopus*, and may contribute as much as 33% of the dry weight of the wall (Bartnicki-Garcia, 1968). Chitosan is a close relative of chitin in two ways: it is physically associated with chitin and is probably derived from chitin by deacetylation.

2.4.2 *Peptidoglycan*

The major structural polymer of virtually all bacterial walls is peptidoglycan (=mucopeptide, murein) which contributes from 5 to 10% (*Escherichia coli*) to 60 to 70% (*Micrococcus luteus*) of the dry weight of the cell wall. Peptidoglycan is similar to chitin in that it is a linear chain made up of β-(1-4) linked acetyl amino sugars but differs in that every other residue is an N-acetylmuramic acid. Short peptides link the parallel chains of amino sugars to form a rigid network. Details of peptidoglycan structure can be found in the review by Rogers (1974).

2.4.3 *Lipopolysaccharides*

Lipopolysaccharides form part of the outer membrane of Gram-negative bacteria and comprise 20 to 30% of the dry weight of the cell wall. As their name suggests they are composed of a polysaccharide and a lipid, yet are extremely varied and complex macromolecules. The lipid fraction consists of β-(1-4) or β-(1-6) linked glucosamine carrying long chain fatty acids (e.g., myristic acid). The lipid is linked, through an eight carbon sugar, to the polysaccharide which contains, in addition to a number of common sugars (glucose, galactose, mannose), L-rhamnose and heptoses.

2.4.4 *Teichoic and teichuronic acids*

Teichoic and teichuronic acids are major cell wall components of Gram-positive bacteria and may constitute 30% to 50% of the dry weight of the wall or about 10% of the total cell. There are several types of teichoic acid but all contain a backbone of polyglycerol phosphate or polyribitol phosphate. In many bacterial species the polyphosphate chain contains sugar and amino sugar substituents. Teichuronic acids are polymers made up of alternating units of uronic acid and a hexose or hexosamine.

The function of these cell wall polyanions is still debated but they may be important in cation binding, regulation of autolytic enzymes, sensitivity to antibiotics, and reception of bacteriophages. Teichoic and teichuronic acids have been reviewed by Duckworth (1977).

Other carbon-containing microbial polymers found in cell walls include mannans (yeasts), lipoproteins, and phospholipids (Gram-negative bacteria), and glycans (filamentous fungi).

2.4.5 *Extracellular polysaccharides*

Several species of bacteria are enveloped in an extracellular layer of polysaccharide. This coat is either of an amorphous nature, in which

case it is described as a slime layer, or has a rather more ordered structure, a capsule. The possible functions of this extracellular polysaccharide have been discussed by Dudman (1977) although it is well to remember that the conditions favouring their production *in vitro* (high nutrient and oxygen levels) may be encountered rarely *in vivo*.

Bacterial exopolysaccharides which have been investigated include: cellulose (produced by certain species of *Acetobacter*), dextrans (*Leuconstoc*), levans (*Pseudomonas, Xanthomonas, Bacillus*), xanthan (*Xanthomonas*), curdlan (*Alcaligenes, Agrobacterium*), alginate (*Azotobacter, Pseudomonas*) and a large variety derived from *Klebsiella, Rhizobium* and *Arthrobacter* species. Pullulan, is produced by the fungus *Aureobasidium*. In many instances detailed structures have not been determined but a number of component sugars have been identified: D-glucose, D-galactose, D-mannose, L-rhamnose, and L-fucose. Some polysaccharides resemble the teichoic acids of Gram-positive bacteria and thus contain glycerol and ribitol, others are composed of the N-acetyl derivatives of D-glucosamine and D-galactose whilst many extracellular polymers consist of uronic acids such as D-glucuronic, D-galacturonic, D-mannuronic, and L-guluronic acid. Powell (1979) has recently described many of these exopolysaccharides.

2.4.6 Cytoplasmic contents

The cytoplasm and cytoplasmic membrane of microorganisms become an important source of carbon-substrates upon the death and lysis of the cell. Phospholipids constitute 30 to 40% of the cytoplasmic membrane of all microorganisms and some yeasts and fungi, contain polymers of mannose and other sugars. All Gram-positive bacterial membranes contain lipoteichoic acid. The cytoplasmic contents of prokaryotes include a plethora of enzymes, substrates and metabolites too numerous to be mentioned here. However, carbohydrate storage materials, notably starch and glycogen, occur in many fungi, yeasts, protozoa, and algae, as well as in species of *Clostridium* and coliform bacteria.

3. MINERALIZATION OF CELLULOSE

3.1 Introduction

The structural polysaccharides of plants, animals, and microorganisms are not easily mineralized: clearly their function is in support and protection. Microorganisms, however, have developed a variety of

polysaccharases and a range of strategies which enable them to overcome the physical and chemical barriers to degradation. Many of these strategies will invoke collections of enzymes often acting in a synergistic manner and either produced by a single species or, more often, by a community of taxonomically diverse microorganisms.

Nonetheless the task faced by microbial polysaccharases is enormous. Even homopolysaccharides, which contain only one type of monomeric unit and are either linear (cellulose) or branched (dextran), present problems to microbes attempting to utilize them as substrates. For instance, they may be poorly soluble, neighbouring chains are stabilized by hydrogen bonds, branch points may retard hydrolysis and non-carbohydrate substituents also affect susceptibility to attack. Heteropolysaccharides, with up to six different monomeric components, may be even more difficult to saccharify. Other constraints on degradation are discussed later in the chapter.

There has been much debate concerned with the early enzymic events in polysaccharide decay. In general, the synergistic activities of two types of extracellular enzymes are implicated: endopolysaccharases and exopolysaccharases. Endopolysaccharases cleave glycosidic linkages within the polymer whilst exopolysaccharases attack linkages to terminal sugar residues. The specificity of these two classes of enzyme is believed to be due to their requirement to bind to other sugar residues or subsites on the polysaccharide during catalysis. For example, exopolysaccharases act more readily as the chain length increases suggesting that a number of sugar residues need to be bound to the enzyme in order to achieve maximum activity. The synergy may be due to the enzymes forming a loose complex with each other and thus optimizing their spatial relationship to the substrate and consequently the rate of hydrolysis. In crystalline structures, such as cellulose, the complex may prevent reformation of ordered zones after endocellulase attack (Wood, 1980). Glycosidases show specificity towards short chains of sugar residues and release monosaccharides.

The mode of action of polysaccharases has been discussed recently by Bacon (1979) as have the methodological problems associated with the enzymology of poorly soluble heterogenous substrates (Lee *et al.*, 1980).

Cellulose is a widespread and abundant source of carbon and thus the possession of enzymes to degrade it is an important attribute of microorganisms. This is particularly true of free-living saprophytic microbes utilizing dead plant tissues, but a cellulolytic capacity may also be essential to pathogenic microorganisms attempting to penetrate plant tissue and to symbiotic microorganisms associated with ruminants and a number of insects.

However, there are a number of physical and chemical constraints on the enzymic degradation of even "pure" cellulose (cotton fibres, filter papers). Some of these have been discussed by Fan *et al.* (1980) and include:

(1) the moisture content of the cellulose fibre—in addition to the obvious requirement for water in hydrolytic events, wet and swollen cellulose presents a greater surface area for enzyme attachment;
(2) the degree of crystallization of the cellulose—the ordered hydrogen-bonded zones of the molecule are far less accessible than the amorphous areas. The degree of crystallinity varies from 50 to 90% according to the type of cellulose;
(3) the conformation and steric rigidity of glucose units;
(4) the degree of polymerization; and
(5) the size and diffusibility of cellulolytic enzymes—in relation to the spaces between the microfibrils and the cellulose molecules. Lower molecular weight endoglucanases (*ca.* 12 500) are suggested as being more effective than their higher molecular weight counterparts (*ca.* 50 000) in the early stages of cellulose depolymerization.

If one now considers cellulose in its native state (plant cell walls), there are a number of additional factors influencing cellulolysis, and in particular the nature of the polymers with which the cellulose is associated. These will be predominantly hemicelluloses, pectin, and lignin in varying proportions depending upon the plant species and its age. Thus, in reality, the cellulolytic microflora must contend with a wide range of heterogeneous substrates with which cellulose forms a chemical and intimate physical relationship. In order to degrade cellulose efficiently the cellulolytic microorganisms must overcome these numerous constraints, a task which could involve one or more of the following strategies:

(i) the ability of an individual species to produce, in addition to its cellulases, a large number of pectinases, hemicellulases and phenolases;
(ii) the capacity to co-operate within a community of microorganisms—some specializing in hemicellulose or pectin decay, others in the depolymerization of lignin;
(iii) the ability to overcome the physical barriers imposed by cell wall constituents by vigorous and sustained growth through regions containing unsuitable substrate; fungi and actinomycetes are said to have this advantage and are viewed by

many as having a key role in the early stages of macroscopic organic matter decay; and

(iv) a capacity to resist the bacteriostatic and fungistatic capacity of phenolics produced during lignin decay and synthesized during humic polymer formation.

The ultimate measure of a truly cellulolytic microorganism is its ability to degrade highly ordered, crystalline cellulose. This is unfortunate from the viewpoint of the enzymologist because crystalline cellulose (as cotton fibres, filter paper, etc.) is insoluble and has a poorly defined and variable structure. Cellulase attack on these substrates is often slow and can only be measured in a semi-quantitative manner by weight loss, change in tensile strength, the release of free fibres, or the solubilization of a dye. Clearly it is impossible to express enzyme activity in terms of standard units or to obtain kinetic data. Frequently, therefore, cellulase activities are assessed using the soluble pseudosubstrates carboxymethylcellulose (CMC) or hydroxyethylcellulose (HEC). However, these two have their limitations in that they also have ill-defined structures and the effect of the substituents upon the enzymes is unknown. As a result CMC-degrading enzymes are often referred to as carboxymethylcellulases (CMCases). It is possible to express activity in absolute units by relating viscosity changes to numbers of broken β-(1-4) bonds (Almin and Eriksson, 1967; Almin *et al.*, 1967). The ability to reduce the viscosity of carboxymethylcellulose is usually regarded as being due to the presence of one or more endoglucanases (EC 3.2.1.4): enzymes which randomly cleave internal 1-4 bonds within the cellulose molecule making it more accessible for subsequent hydrolysis. There are no specific substrates with which to measure exocellulase activity and thus for direct measurements a purified enzyme is necessary. However, the production of reducing sugars from an amorphous cellulose reflects the combined activities of endoglucanase and exoglucanase and the difference between this result and CMCase activity (only due to endoglucanase) gives a measure of exoglucanase. According to Wood and McCrae (1978b) a rapidly acting endoglucanase would be expected to produce a rapid decrease in viscosity in relation to the appearance of reducing sugars (i.e., high ratio of viscometric change to reducing sugars); a less rapidly acting endoglucanase or an exoglucanase would produce a lower ratio.

β-(1-4)-Glucosidases hydrolyse cellobiose and short chain cello-oligosaccharides to glucose. Various simple and reliable assays are available using cellobiose, salicin and the pseudosubstrates *o*-nitro-

phenyl-β-D-glucanopyranoside (ONPG) and *p*-nitrophenyl-β-D-glucopyranoside (PNPG).

Techniques for measuring cellulases have recently been assessed by Eriksson and Johnsrud (1982).

3.2 Axenic Culture Studies

3.2.1 Fungi

The ubiquitous soil hyphomycete genus *Trichoderma* has been the most widely studied cellulolytic fungus (Mandels, 1975; Pettersson, 1975; Enari and Markkanen, 1977; Wood and McCrae, 1978a). This is due to fact that the two principal species, *T. viride* and *T. koningii*, are capable of synthesizing and secreting the entire cellulase complex. In other words, the distinguishing feature of the *Trichoderma* cellulase system is that it contains extracellular endocellulases, exocellulases and β-glucosidases which work in concert to solubilize highly-ordered cellulose. *Trichoderma* species share this ability with a few other fungi, notably *Fusarium solani* (Wood and Phillips, 1969), *Sporotrichum pulverulentum* (Eriksson and Rzedowski, 1969) and *Penicillium funicolosum* (Wood and McCrae, 1978b) but cell-free culture filtrates from most other cellulolytic fungi and bacteria only effect some of the hydrolytic steps *en route* to glucose. This fact suggests that the successful depolymerization of cellulose in many environments will demand the co-operative efforts of more than one microbial species.

The starting point for all theories of cellulose depolymerization is the C_1-C_x hypothesis of Reese *et al.* (1950). They proposed that the native cellulose was first attacked by an extracellular non-hydrolytic enzyme (C_1) which loosened the fibrils by breaking the hydrogen bonds holding the cellulose chains together. This, in effect, increased the accessibility of the substrate to the subsequent activities of hydrolytic enzymes (C_x). Although this model has proved inadequate, and sometimes misleading, many research workers still attempt to relate their findings to it. Thus the C_1 enzyme is viewed by many as a specific exoglucanase, cellobiohydrolase (Wood and McCrae, 1972; Berghem and Pettersson, 1974; Halliwell and Griffin, 1978) whilst the C_x enzymes are equivalent to endoglucanase. Therefore, if a serial process of saccharification is countenanced it is more likely to be a C_x-C_1 sequence; the reverse of that originally proposed. However, although it appears logical that endoglucanases initiate cellulose degradation, it may be more useful (Wood and McCrae, 1972) to envisage a number of parallel and synergistic enzymic events occurring during the solubilication of crystalline cellu-

lose. The original notion of a non-hydrolytic, but so far unidentified, disaggregating enzyme is still not discounted by some (Leatherwood, 1969; Reese, 1977), and the subject of a preliminary enzymic disaggregation or disturbance of cellulose continues to be warmly debated (Reese, 1977; Wood, 1980; Lee and Fan, 1980).

Berghem *et al.* (1975, 1976) have isolated and purified four cellulases from *Trichoderma viride*. These include two endoglucanases capable of attacking the internal bonds of carboxymethylcellulose. Endoglucanase I has a molecular weight of 12 500 and is more active than endoglucanase II (M.W. 50 000) at releasing free fibres from cotton and filter paper. It is possible that its lower molecular size increases its penetration of the insoluble cellulose molecule. A single exoglucanase (M.W. 42 000) was also isolated and was extremely efficient at removing cellobiose but not glucose units from the non-reducing ends of microcrystalline cellulose (Avicel). This enzyme was also somewhat active against cotton and was product inhibited. Others have reported more than one form of exoglucanase in *T. viride* (Gum and Brown, 1977; Gritzali and Brown, 1979). The fourth enzyme reported by Berghem *et al.* (1975, 1976) was a β-glucosidase (M.W. 47 000) which cleaved cellobiose, various higher oligosaccharides (e.g. cellotetraose), and the pseudosubstrate *p*-nitrophenyl-β-D glucoside. Not surprisingly when the exoglucanase and the β-glucosidase are combined degradation of Avicel was accelerated because of the removal of cellobiose. The β-glucosidase is associated with the cell fraction (Berg and Pettersson, 1977)—a common location in other cellulolytic microorganisms. Incidentally, endoglucanase may exhibit activity towards cellobiose (Ladisch *et al.*, 1980) and is product inhibited.

Using *Trichoderma koningii*, Wood and McCrae (1978a) identified four major endoglucanases with molecular weights ranging from 13 000 to 48 000. By examining various combinations of these endoglucanases with the cellobiohydrolase from the same species they were able to propose that the efficiency of the enzymes was related to their ability to form endoglucanase-cellobiohydrolase complexes on the surface of the cellulose chain. Thus something more than a casual synergy is envisaged in cellulose breakdown. In addition, cellobiose was recorded as being strongly inhibitory to three of the four endoglucanases and also to the cellobiohydrolase. This may be a limiting factor in cellulose hydrolysis for many *Trichoderma* strains as β-glucosidase is produced in low amounts relative to the endo- and exoenzymes (Sternberg, 1976). In general all reactions are product inhibited, usually by competitive inhibition (Halliwell *et al.*, 1972; Howell and Stuck, 1975; Sternberg, 1976), although glucose is a non-competitive inhibitor of cellobiase in *Trichoderma viride* (*T. reesei*) (Ladisch *et al.*, 1980).

Sophorose is the most potent inducer of cellulases in *Trichoderma* spp. (Sternberg and Mandels, 1979) although the natural inducer is probably the somewhat less effective cellobiose (Mandels *et al.*, 1962). Of course, sophorose is itself hydrolysed intracellularly to glucose which then acts as a repressor of cellulase formation (Nisazawa *et al.*, 1972; Loewenberg and Chapman, 1977). Inglin *et al.* (1980) isolated an intracellular β-glucosidase (M.W. 98 000) from *Trichoderma viride* (*T. reesei*) that hydrolysed both sophorose and cellobiose and was distinct from the previously-isolated extracellular β-glucosidases (Berghem and Pettersson, 1974; Gong *et al.*, 1977). They speculated that the intracellular β-glucosidase controls cellulase induction by destroying the inducer and producing a repressor. Gritzali and Brown (1979) have recently shown that sophorose will cause *T. viride* (*T. reesei* strain QM9414) to excrete endoglucanases into the growth medium.

Cellulases of the white-rot fungus *Phanerochaete chrysosporium* (= *Chrysosporium lignorum* = *Sporotrichum pulverulentum*) have been extensively studied by Eriksson and co-workers (Eriksson and Rzedowski, 1969; Almin *et al.*, 1975; Deshpande *et al.*, 1978; Eriksson, 1978). They have reported the presence of five endoglucanases, one exoglucanase (releasing cellobiose or glucose—cf. *Trichoderma* sp.) and two β-glucosidases and have demonstrated a strong synergistic response between the endo- and exoglucanase components. Two other non-hydrolytic enzymes were also described as part of the cellulase complex: a cellobiose oxidase (M.W. 100 000) converting cellobiose to cellobionic acid (Ayers *et al.*, 1978) and cellobiose-quinone oxidoreductase (CBQ), which also oxidizes cellobiose. CBQ is implicated in the degradation of lignin (Westermark and Erikkson, 1975) and will be discussed later in this chapter. *Polyporus versicolor* also produces CBQ enzyme but neither cellobiose oxidase nor cellobiose-quinone oxidoreductase had, until recently, been described for *Trichoderma* sp. However, Vaheri (1980) has implicated oxidative enzymes in cellulose breakdown by *Trichoderma reesei* (formerly *T. viride* strain QM 6a). The regulation of cellulases in *Phanerochaete chrysosporium* has been discussed by Eriksson(1979) who has commented upon the number of differences in this system when compared to *Trichoderma viride*. Smith and Gold (1979) have reported that *Phanerochaete chrysosporium* produces intracellular soluble and particulate β-glucosidases as well as an extracelluar β-glucosidase. The extracellular enzyme (MW 90 000; pH optimum 5.5) is induced by cellulose but repressed by glucose. In contrast, the intracellular enzyme (MW 410 000; pH optimum 7.0) is induced by cellobiose and not affected by glucose.

Penicillium funiculosum when grown on cellulose as the sole carbon and energy source, secretes enzymes that are active against all forms of

cellulose (Selby, 1968; Wood and McCrae, 1977). Recently Wood *et al.* (1980) have isolated the sole cellobiohydrolase (M.W. 46 000) from this fungus and found that, although it was capable of hydrolysing phosphoric acid-swollen cellulose, it had little activity against highly ordered celluloses (e.g. cotton). However, when recombined in the original proportions with its endoglucanases (three or more) and β-glucosidases (two or three), 98% of the original activity against this substrate was recovered. Synergistic activity was also observed when the *Penicillium* sp. cellobiohydrolase was mixed with endoglucanases from other fungal sources, notably *Trichoderma koningii* and *Fusarium solani* (Wood *et al.*, 1980), and this discovery is discussed elsewhere in this chapter.

Many other fungi have been investigated for their cellulolytic activities. Germinating microcysts of the cellular slime mould, *Polysphondylium pallidum*, excrete endocellulase (CMCase) and β-glucosidase during the emergence of amoeba (O'Day and Paterno, 1979). However, this is essentially a cyst cell wall digesting system and the cellulases may have little effect once they have passed through the cell wall. *Rhizoctonia lamellifera* exhibits both endocellulase and cellobiase activities enabling it to grow on a range of soluble and insoluble cellulose substrates (Selby and Maitland, 1967; Olutiola and Ayers, 1973; Olutiola, 1976). The white rot fungi are particularly important in cellulose decay *in vivo* because of their involvement in lignin degradation (Lundquist *et al.*, 1977).

The cellulases of the thermophilic fungus *Talaromyces emersonii* have been studied by Folan and Coughlan (1978) and those of *Sclerotium rolfsii* have been investigated by Shewale and Sadana (1978, 1979). The latter Basidiomycete fungus secretes high amounts of cellulases, including cellobiase, which are not inhibited by the presence of cellobiose and glucose. Other Basidiomycetes which have been studied in this context include *Schizophyllum commune* (Wilson and Niederpruem, 1967), *Lenzites trabea* (Herr *et al.*, 1978), *Stereum sanguinolentum* (Bucht and Eriksson, 1969) and *Stachybotrys atra* (Jermyn, 1967).

3.2.2 Bacteria

The cellulose-degrading capacity of the genus *Cytophaga* has been known for many years (Walker and Warren, 1938). More recently Chang and Thayer (1977) have used this flexibacterium to investigate the activities and functional location of the various enzymes of the cellulase complex. They found that in crude extracts cellulases

(measured against carboxymethylcellulose) were located either on or within the cytoplasm and periplasm, and that the membrane-bound component reduced CMC viscocity whilst the soluble fraction produced soluble sugars. Cell-free endocellulases were not found. The cytoplasmic endocellulase had a molecular weight of 8650 whereas the periplasmic equivalent had a molecular weight of 6250. In addition to the CMC-hydrolysing endoglucanase, the periplasmic fraction contained an exocellulase, active against microcrystalline cellulose, and most of the bacteria's β-glucosidase activity.

Another flexibacterium, *Sporocytophaga myxococcoides* has been described as one of the most active cellulolytic microorganisms, perhaps because it is able to penetrate fibres to obtain maximum substrate-cell contact. Thus, it degrades insoluble cellulose fibres by direct contact and was originally seen under the electron microscope by the appearance of cavities at contact sites (Berg *et al.*, 1972b). It produces several endoglucanases active against CMC (Osmundsvag and Goksøyr, 1975). Recent studies by Vance *et al.* (1980) have shown that *S. myxococcoides* produces an extracellular CMCase and activity towards Avicel (an exocellulase). The former observation confirms the work of Berg *et al.* (1972b), whereas the latter is a contradiction.

Beguin and Eisen (1978) purified three extracellular endocellulases from cultures of *Cellulomonas flavigena*; one free in solution, the other two bound to the substrate (cellulose powder). The soluble endocellulase had a molecular weight of 118 000 and the two bound ones were around 50 000. The two bound enzymes were glycosylated which is also a feature of cellulases from other microbial sources (Berghem *et al.*, 1976; Erikkson and Pettersson, 1975). The authors state that these three enzymes do not constitute the total cellulolytic complex of *C. flavigena*; indeed at least two other CMC-ases were present in the growth medium. All enzymes were subject to repression by readily assimilatable carbon sources and, in the absence of cellulose, cellobiose and even sophorose proved to be weak inducers (Beguin *et al.*, 1977). Stewart and Leatherwood (1976) have obtained mutants of a *Cellulomonas* sp. which do not exhibit catabolite repression.

The capacity of *Cellvibrio* sp. to degrade various forms of cellulose fibre depends upon the structure and complexity of these fibres (Berg *et al.*, 1972b). However, there is no doubt that members of the genus *Cellvibrio* produce cellulases capable of attacking crystalline cellulose (Berg *et al.*, 1972a). Berg (1975), studying *Cellvibrio vulgaris*, found that the location of cellulases depended upon the carbon source and the age of the culture. When cells were grown on soluble carbon (i.e. cellobiose or glucose) all the CMCase was cell-bound (i.e. associated with the

periplasm and on the cell surface). Growth on cellulose resulted in cell-free CMCase. Oberkotter and Rosenberg (1978, 1980) also observed the active secretion of endoglucanases into the culture medium during exponential and maximum population phases of growth.

Themophilic actinomycetes have long been known as dominant mineralizing microorganisms in compost and other high temperature environments which contain organic debris (Hankin *et al.*, 1976). For instance, *Thermomonospora curvata* is just one cellulolytic prevalent in compost (Stutzenberger, 1972).

Su and Paulavicius (1975) using a *Thermoactinomyces* sp. indicated that two β-glucosidases were secreted into the culture medium and that they had different thermal stabilities. In contrast, Hagerdal *et al.* (1978, 1979) using the same genus, provided evidence that the β-glucosidase component was exclusively a soluble intracellular enzyme and that the culture filtrate contained other cellulases (e.g. cellobiohydrolase). This work in effect supports the view of Mandels (1975) that the culture filtrate of *Thermoactinomyces* sp. is incapable of complete cellulose hydrolysis, although she believed that it was the cellobiohydrolase that was absent.

The general model of cellulose breakdown by *Thermoactinomyces* species (Humphrey *et al.*, 1977) is essentially that proposed for the fungi. Namely one or more endoglucanases release oligosaccharides which are hydrolysed by exoglucanases to produce cellobiose or glucose. Several β-glucosidases exist which are able to cleave the cellobiose.

Enzymes of the mesophilic cellulolytic actinomycete, *Streptomyces flavogriseus*, have been described by Ishaque and Kluepfel (1980). This species when grown on Avicel, produced considerable amounts of extracellular endoglucanase whilst β-glucosidase and cellobiase activities were predominantly associated with the mycelial fraction and could only be released by sonication. The actinomycete displayed good overall cellulolytic activity towards filter paper and cotton and the enzymes had an enhanced stability in the presence of the substrate.

Cellulases have been recorded in the thermophilic bacterium *Clostridium thermocellum* (Lee and Blackburn, 1975; Weimer and Zeikus, 1977). Ait *et al.* (1979) isolated an extracellular complex (M.W. 125 000) from this anaerobe which expressed CMCase and cellulase activities and contained carbohydrate residues. The authors suggested that the cellulases bind to cellodextrins produced during the hydrolysis of cellulose. Saddler and Khan (1979) isolated from sewage sludge a member of the Bacteriodaceae (probably *Bacteriodes succinogenes*) which produced a cellulase complex degrading cellulose to cellobiose (an

endo-exo system) and showed that the cellobiose was utilized fermentatively producing hydrogen, carbon dioxide, glucose, and acetic acid.

There are a number of anaerobic cellulolytic bacterial genera, many of them isolated from ruminant animals, e.g., the genera *Bacteriodes* and *Ruminococcus* (Halliwell and Bryant, 1963; Hungate, 1966) or from cellulose-digesting insects (Thayer, 1978). The anaerobic digestion of cellulose has recently been discussed by Scharer and Moo-Young (1979). An unidentified *Pseudomonas* species secretes extracellular endoglucanases (as does *P. fluorescens* var. *cellulosae*—Yamane *et al.*, 1970, 1971) and has cell-bound endoglucanase and β-glucosidase activity (Bevers, 1976; Hwang and Suzuki, 1976).

Ramasamy and Verachtert (1980) also studied the cellulases of a *Pseudomonas* species. Two endoglucanases were found in the culture medium during growth on cellulose but not on cellobiose, although cell-bound endoglucanases were present with both substrates. Three cell-bound β-glucosidases were detected, two in the periplasmic space and one in the cytoplasm. The more external of the periplasmic glucosidases increased its concentration when grown on cellobiose. These authors conclude that the location (and, not surprisingly, the concentration of enzyme) is determined by the particular substrate: endoglucanase was more external for growth on cellulose than for growth on cellobiose. As occurs with *Trichoderma* spp., sophorose induces cellulase synthesis in *Pseudomonas* spp. (Suzuki *et al.*, 1969; Yamane *et al.*, 1970).

3.3 Community Studies

We have already seen that the total breakdown of cellulose demands the combined efforts of a number of different enzymes and that the various microbial species involved are not equally effective. Some fungi, such as *Trichoderma* spp., have the capacity to produce the complete compliment of cellulases necessary to convert a highly ordered cellulose (e.g. cotton fibre, filter paper) into glucose. Many bacteria, however, find it difficult or are totally unable to degrade native cellulose, yet are active against pre-treated cellulose or soluble derivatives. A considerable number of microbial species, though not producing endo- or exoglucanases (and therefore not considered to be truly cellulolytic), have β-glucosidase activity and are capable of growing on the soluble, low molecular weight products of cellulolysis. The inability of individual microbial species to degrade cellulose may be due to many factors:

(i) the total absence of certain essential cellulolytic enzymes;
(ii) the absence or incorrect concentration of inducer molecules;
(iii) the presence of enzyme repressor or inhibitor molecules;
(iv) the relationship between substrate adsorption and desorption of the various cellulases such that the optimum ratios of active components are not maintained;
(v) the inability of the cell to locate itself in close proximity to the substrate—crucial for those with cell-bound cellulases and suggesting chemotaxis;
(vi) the absence of enzymes whose function is to release the cellulose from its cell-wall associates (hemicellulose, lignin); and
(vii) the lack of a capacity to overcome the bacteriostatic and fungistatic properties of certain components of the organic debris.

Taking these constraints into account, it is plain to see that if it retains its independence, a high proportion of the cellulolytic microflora will have enormous problems in degrading native cellulose in natural environments. The obvious strategy, therefore, and one which may be frequently adopted, is to form a mutually beneficial association with one or more different microbial species.

One approach to understanding multiple interactions in the breakdown of cellulose is to monitor the cellulose-degrading properties of known permutations of cellulases taken from the growth medium of different microbial species. This type of experiment simplifies the investigation by eliminating inter-microbial interaction along with problems associated with induction and suppression and merely studies the enzymic capacity of the system. It is well known that many *Trichoderma* strains produce insufficient β-glucosidase for the rapid saccharification of cellulose. Sternberg *et al.* (1977) screened a number of fungi for β-glucosidase activity and found that two *Aspergillus* species (*A. niger* and *A. phoenicis*) were superior producers. When *Trichoderma* spp. cellulase preparations were supplemented with *Aspergillus* spp. β-glucosidase, the rate of cellulolysis (of Avicel and purified wood cellulose) increased and glucose was the major product. The co-operative and synergistic effects of cellulolytic enzymes from different sources has also been demonstrated in an extensive study by Wood *et al.* (1980). These workers measured the solubilization of cotton cellulose by mixtures of cellobiohydrolase (contained in culture filtrates of *Penicillium funiculosum*) with endoglucanases from a number of other fungi. Table 2 reveals that synergism was apparent in all combinations but was most evident when cellobiohydrolase was mixed with the endoglucanases of *Trichoderma koningii* or *Fusarium solani*. Significantly it is these two species that normally release a cellobiohydrolase (although

Table 2. Cotton-solubilizing activity of *Pencillium funiculosum* cellobiohydrolase in mixtures of endo-β (1-4) glucanases produced by other fungi. (From Wood *et al.*, 1980).

	Solubilization of Cotton (%)	
Source of Endoglucanase	Endoglucanase alone	Endoglucanase + *P. funiculosum* cellobiohydrolase
Trichoderma koningii	1	51
Fusarium solani	1	45
Myrothecium verrucaria	6	20
Stachybotrys atra	4	11
Memnoniella echinata	5	18

previously removed in this experiment) into the culture medium along with the endoglucanase. The other three fungi only release the endoglucanases. According to Wood (1980) the variation in co-operative action between the two cellulases may be due to the stereochemical requirements of their active sites or the need to form a cellulase complex on the surface of the cellulose crystallite before hydrolysis can take place. Since this experiment was concerned with the combined activities of extracellular cellulases and since it is apparent that cellulose can be degraded in this manner, it is not difficult to envisage combinations of two or more of these fungi co-operating in the natural environment. Certainly one will often isolate species of *Penicillium*, *Trichoderma*, *Fusarium*, and *Stachybotrys* from the same soil sample. The author, however, is unaware of any detailed studies concerning cellulose degradation by a defined community containing two or more of these fungi. Indeed, there have been few detailed investigations of any microbial communities involved in aerobic cellulose mineralization—other than those with an ecological bias described on p. 506. Recently, however, Lynch *et al.* (1980) have looked at microbial populations concerned with the breakdown of a crude cellulose fraction extracted from straw. Their preliminary studies implicated an eleven-membered community comprised of seven fungi (including a *Trichoderma* sp., *Aspergillus* spp., a *Mucor* sp., and a *Penicillium* sp.) two yeasts and two bacteria (one an actinomycete). The yeasts and bacteria did not exhibit cellulase activity but were probably contributing to cellulose breakdown by depolymerizing the associated xylan and arabinan which represented

11% of the cellulose extract. Possible communal relationships in cellulose decay are depicted in Fig. 1.

Some of the most convincing data implicating microbial communities in cellulose mineralization have come from investigations of anaerobic environments. The anaerobic fermentation of cellulose and other sugar polymers to produce methane and carbon dioxide is an essential component of carbon mineralization in marshes, peat bogs, and aquatic sediments as well as in sewage digesters and the ruminant stomach. There is no doubt that the entire sequence involves a microbial community composed of as many as four physiologically-distinct groups (Wolfe, 1979): one group effecting the breakdown of cellulose and fermenting the products to fatty acids, carbon dioxide, and hydrogen; another concerned with the conversion of fatty acids to acetate and hydrogen; a third oxidizing hydrogen to acetate with the reduction of carbon dioxide; and a fourth group converting acetate, hydrogen and carbon dioxide, methanol, and formate to methane. The number of groups and particular species concerned will be determined by the source of cellulose, pH, temperature, and other factors. In sediments, marshes, bogs, and sewage digesters, all four groups have been implicated. In the rumen the fatty acids are absorbed into the blood stream and are not converted to acetate and hydrogen. In fact in the rumen (and unlike other anoxic environments) methane does not arise from acetate.

Perhaps the best evidence for the involvement of microbial communities in the anaerobic mineralization of carbon polymers has come from studies of the rumen (Bryant, 1977; Hobson and Summers, 1978; Hobson, 1979). Clearly bacteria which produce a

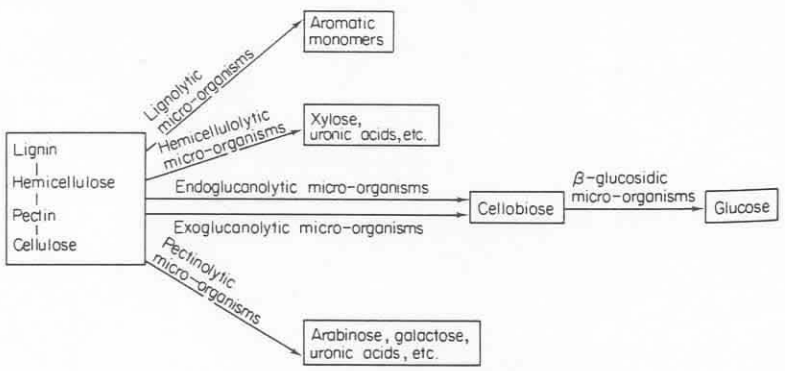

Fig. 1. Possible communal relationships between microorganisms during native cellulose depolymerization.

variety of polysaccharases are well-prepared for the decay of whole plant material as it enters the ruminant stomach. Indeed, there are a number of rumen bacteria which ferment hemicellulose constituents and pectins as well as cellulose (e.g. *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Butyrivibrio fibrisolvens*). Others, such as *Bacteriodes succinogenes*, will degrade cellulose but not xylan whilst a few can only utilize starch (e.g. *Selenomonas ruminantium*, *Bacteriodes amylophilus*). Perhaps surprisingly some bacteria may solubilize xylans and pectins without being able to ferment the resulting oligosaccharides, possibly relying upon associated cellulolytic microorganisms to provide a suitable carbon source. A synergistic relationship between a non-cellulolytic but hemicellulolytic bacterium (*Bacteriodes ruminicola*) was shown to increase cellulose breakdown by a cellulolytic bacterium (Dehority and Scott, 1967). Incidentally, Coen and Dehority (1970) reported that a two-membered community composed of *Bacteriodes ruminocola* and *Ruminococcus flavefaciens* could totally mineralize grass hemicellulose whereas axenic cultures could only perform a portion of the sequence. Scheifinger and Wolin (1973) demonstrated that *Selenomonas ruminantium* and *Bacteriodes succinogenes* grew well in mixed cultures with cellulose as their sole energy source. *S. ruminantium*, however, is non-cellulolytic and thus will not grow on cellulose, although it will utilize glucose—a possible metabolite in the mixed culture. The selenomad certainly produced propionate from the carbohydrates as well as from the decarboxylation of any succinate. Unfortunately, cross-feeding supported by the soluble products of polymer hydrolysis is as poorly understood in the rumen as in other environments, and the success of non-polysaccharase producers may be due to the presence of a range of fermentation products or even cell debris.

Dilworth *et al.* (1980) have recently summarized their investigations of reconstituted anaerobic mesophilic cellulolytic communities. In one experiment, mixed cultures containing cellulase producers (primary bacteria) and cellobiose fermenters (secondary bacteria) were grown at 37°C for 25 d in the presence of cellulose as a sole carbon source. Whereas neither the primary nor the secondary bacteria grew rapidly on cellulose in pure culture, the community utilized 48% of the substrate during the course of the experiment and produced hydrogen, carbon dioxide, ethanol, and acetate. The secondary bacteria were almost certainly accelerating cellulolysis by removing cellobiose which is known to repress cellulase synthesis and activity. A nutritional synergism was not suggested. In addition, the cellobiase producer was the major influence as far as the products of cellulose fermentation were concerned. In another experiment a different secondary bacterium

(identified as *Clostridium glycocolum*) was used in the community with the result that acetate was the major product to the exclusion of hydrogen and ethanol. *C. glycocolum* grown axenically on cellobiose yielded large amounts of acetate.

Bacterial methanogenesis represents the final stage of biopolymer mineralization in anaerobic environments. Dilworth *et al.* (1980) have described the conversion of cellulose to methane in mixtures of primary, secondary and hydrogen-utilizing methane bacteria. The combination of cellulolytic species was again required for cellulose degradation but the methanogenic species (*Methanobacterium* sp.), although growing successfully, only marginally stimulated fermentation and should be considered as a commensal component of the community. However, when the *Methanobacterium* species was replaced by *Methanosarcina barkerii*, a more significant increase in the rate of cellulose utilization was seen. Experiments with other combinations of microorganisms indicated that *M. barkerii* stimulated growth of cellulolytic organisms by removing inhibitory fatty acids, such as acetate or butyrate, and not by utilizing cellobiose or hydrogen.

The resolution of the symbiotic association "*Methanobacterium omelianskii*" (Bryant *et al.*, 1967) has led to a greater understanding of the interactions between methanogens and heterotrophic anaerobes. Interspecies hydrogen transfer, although not demonstrated in the mixtures described above, has been suggested as an important factor in the stimulation of fermentation rates in mixed cultures (Bryant *et al.*, 1977). In this process the hydrogen-evolving bacteria directly support the hydrogen-utilizing methanogenic bacteria and in return the methanogens serve as electron sinks allowing the cellulolytic bacteria to dispose of electrons as hydrogen rather than as other reduced and possibly inhibitory products (e.g. ethanol). Iannotti *et al.* (1973) studied the interaction of a mixed culture of rumen anaerobes in continuous culture and discovered that the growth of *Vibrio succinogenes* depended upon the hydrogen produced from glucose by *Ruminococcus albus*. Weimer and Zeikus (1977) grew the thermophilic anaerobes *Methanobacterium thermoautotrophicum* and *Clostridium thermocellum* together in batch culture with cellulose as their source of carbon. The presence of the methanogenic species in the mixture altered the fermentation pattern of the *Clostridium* species by causing a shift in the conversion of acetyl-CoA from ethanol to acetic acid. The result was that more electrons became available for the production of hydrogen which was then utilized by *M. thermoautotrophicum*—the methanogen acting as an "electron sink".

Other syntrophic communities reported to be involved in the anaerobic mineralization of cellulose, cellobiose and glucose to methane

and carbon dioxide include: *Acetobacter woodii* and *Methanosarcina barkerii* (Winter and Wolfe, 1979); *Ruminococcus flavefaciens* and *Methanobacterium ruminatium* (Latham and Wolin, 1977); and *Citrobacter* sp. and *Methanobacterium fromicicum* (Sineriz and Pirt, 1977).

4. MINERALIZATION OF NATIVE POLYSACCHARIDES

4.1 Introduction

Microorganisms must contend with a large number of polysaccharides in addition to cellulose. These will include comparatively simple structures, such as starch, chitin, dextran, and glucans; complex heteropolymers, such as hemicellulose, pectin, and teichoic acids; and ill-defined polysaccharides, such as gums and mucilages.

The same general principles which govern microbial and enzymic co-operation during cellulose mineralization can be applied to all polysaccharides. Thus, as previously described for cellulose, a sequence of events is envisaged during which various microbial species contribute towards the total decay of a particular substrate. The number of species (and enzymes) involved and the rate of the reaction will be determined by structural and chemical features of the substrate, such as linear or branched chains, homo- or heteropolymers, molecular size, types of bonds connecting the monomers, type and rate of substitution, as well as the substrate's relationship with the other cell constituents.

Not surprisingly, attempts to understand the mineralization of other polysaccharides have paralleled those detailed for cellulose. Unfortunately, however, the literature is not extensive and there is little doubt that the same stimuli that have encouraged the study of cellulose have tended to deflect interest away from polymers such as starch, pectin, chitin, and the hemicelluloses. However, there are studies of axenic cultures and the action of isolated enzymes against pure substrates, and some attempts have been made to understand the decay of plant and microbial debris. The former aspect has been reviewed recently (Sturgeon, 1979a,b; Kennedy, 1979; Dekker, 1979; Manners, 1979; Pilnik and Rombouts, 1979) the latter is considered within this section.

There have been a number of studies of the degradation of ill-defined polysaccharide-containing substrates, including plant materials (lignocellulose, straw, leaf litter, wood, lignin), microbial tissues (entire cells, membranes, cytoplasm) and manufactured products (newsprint, cardboard, paper tissue). Many of these investigations have again

focused on the cellulose component and have fallen into two broad areas. The first is concerned with the substrate as a renewable resource and concentrates on maximizing fermentation products and biomass production; the second involves the microbial ecology of substrate colonization and succession. There is a tacit agreement in these diverse approaches that the breakdown of organic debris is likely to be a multi-stage, multi-organism process and thus much of this work demands (yet may not receive) the study of dynamic microbial communities.

4.2 Plant Organic Matter

4.2.1 Lignin and lignocellulose

It is clear that the presence of lignin in virtually all mature plant materials is a severe handicap to many polysaccharase-producing microorganisms. Not only does lignin form a mechanical barrier against microbial and enzymic penetration (Kirk, 1975) but some of its aromatic constituents may be bacteriostatic and fungistatic, whilst others may selectively inhibit certain carbohydrate depolymerizing enzymes. For instance, Varadi (1972) demonstrated that a number of phenolic substances (e.g. *p*-hydroxybenzyl alcohol, vanillin, syringaldehyde, *p*-coumaric acid, cinnamic acid) repressed the production of cellulase and xylanase in the fungus *Schizophyllum commune* and recently Vohra *et al.* (1980) reported that ferulic acid reduced β-glucosidase activity to zero whilst partially inhibiting endo- and exocellulase activity. Thus it may be realistic to view lignin degradation as the primary rate-limiting step in polysaccharide mineralization. Certainly pretreatment of organic matter to remove lignin accelerates the subsequent decay of residual polysaccharides (Chahal *et al.*, 1979).

Most studies of the breakdown and enzymology of lignin have involved the white-rot Basidiomycetes (e.g., *Coriolus versicolor, Phanerochaete chrysosporium, Pleurotus ostreatus*) all of which are able to mineralize the substrate totally to carbon dioxide. However, the details are far from conclusive and many questions about lignin decay remain unanswered. To a large extent the very nature of the lignin polymer creates problems in that, in contrast to the polysaccharides, it does not contain repeating units nor are the linking bonds easily hydrolyzed. Indeed, hydrolases are probably of little importance in lignin depolymerization. In addition, lignin is chemically heterogenous and large quantities of a standardized lignin substrate are not easily available. Traditionally heterogeneous substrates, such as milled wood lignin or lignin derived from pulping operations (kraft lignins, lignin sulphonates), have been used but in recent years [^{14}C]-labelled lignins

have become available, sensitive and definitive assays have been developed and considerable progress made in describing the events which occur during degradation (Kirk *et al.*, 1977).

The white-rot fungi may attack the lignin polymer in a number of ways:

(i) oxidation of propoid side chains to yield aromatic residues;
(ii) cleavage of β-aryl ether linkages; or
(iii) oxidative cleavage of aromatic rings still attached to the polymer.

The low molecular weight aromatic structures produced by these activities (e.g., vanillin, vanillate, syringaldehyde, guaiacylglycerol, *p*-hydroxybenzoate, coniferaldehyde, *p*-coumarate) are mineralized intracellularly by a large number of fungal and bacterial species (Cain, 1980) or are converted into novel humic polymers (Martin and Haider, 1980). Intriguingly an extracellular aromatic ring cleavage is suggested although how the coenzyme-requiring mono- and di-oxygenases can function has not been revealed.

Lignin breakdown is an obligately aerobic process (Hackett *et al.*, 1977) even though its metabolites are substrates for both aerobic and anaerobic microorganisms. As mentioned previously, the inter-monomer linkages of lignin are not attacked by hydrolases but require oxygenases (Dagley, 1978). Clearly oxygen has a mechanistic role in lignin decay but its exact function is uncertain. Two possibilities discussed by Zeikus (1980) and Shimada (1980) are the need to convert ether-containing linkages to esters prior to hydrolysis and the requirement for superoxide radicals. The latter may even contribute to the chemical degradation of lignins.

The enzymes participating in lignin metabolism have yet to be unequivocally identified and described. No doubt part of the problem is the difficulty of obtaining specific lignolytic activities in cell-free preparations and it is probable that lignases are cell wall bound and unstable when released and that direct contact between hyphae and substrate is required for degradation (Rosenberg, 1978). Nevertheless, there is no shortage of speculation concerning lignin enzymology and one group of enzymes, the phenol oxidases, are frequently implicated (Freudenberg and Neish, 1968). Phenol oxidases may have a variety of functions: demethoxylation, α-carbonyl oxidation, side-chain elimination, and polymerization. More specifically, *o*-demethylase catalyses the demethylation of lignin to yield formaldehyde, and the commonly produced laccase stimulates demethoxylation and methanol production. However, some 80% of the lignin phenolic groups are esterified and therefore cannot be substrates for laccase. Incidentally, laccases pro-

duced by the white-rot genus *Trametes* have been implicated in the polymerization of coniferyl alcohol to lignin. Peroxidase will also cause substantial demethoxylation in some cases. It has been suggested (Ander and Eriksson, 1976) that phenol oxidases may have a regulatory role in cellulose (and lignin) degradation in that they destroy phenols which are potential enzyme inhibitors. Cellobiose: quinone oxidoreductase (CBQ) is an extracellular enzyme which has been found in culture filtrates of *Polyporus versicolor* and *Phanerochaete chrysoporium* (Westermark and Eriksson, 1974, 1975), and *Sporotrichum* (*Chrysoporium*) *thermophile* (Canevascini and Meier, 1978) and is believed to have a dual role in lignin and cellulose decay. This enzyme which, according to Westermark and Eriksson (1975), has a molecular weight of 58000, reduces quinones (produced by the activity of phenol oxidases) to catechols, and simultaneously oxidises cellobiose to cellobiono-σ-lactone (cellobionic acid). The catechols are known to be suitable substrates for the dioxygenases responsible for ring cleavage.

There has been some effort directed towards determining the role of microorganisms other than the white-rot Basidiomycetes in lignin biodegradation. Certainly bacteria are able to degrade a wide range of low molecular weight (<500) lignin-related aromatic monomers and dimers and many of the pathways may be plasmid-encoded. However, studies of bacterial degradation of the lignin macromolecule have not been conclusive and even if it occurs the rate is slow compared to that achieved by the white-rot fungi (Haider and Trojanowski, 1980). Yeasts can grow on di-lignols as can a range of common soil Fungi Imperfecti (*Fusarium*, *Aspergillus*, *Penicillium*) yet no degradation of native lignin has been reported. The lignolytic activity of brown-rot fungi is limited to demethylation and some oxidation reactions (Kirk, 1971). Probably the greatest, and as yet unfaced, challenge to those investigating lignin decay is to understand the activities of such a diverse microflora for there is little doubt that the mineralization of lignin in natural environments proceeds slowly and is achieved by the combined and sequential activities of a variety of organisms. Figure 2 illustrates one possible series of events in lignin mineralization.

Due to the intimate relationships between lignin and glucans most naturally-occurring plant-derived substrates are referred to as lignocelluloses. By their very nature lignocelluloses are non-reproducible substrates although some have attempted standardization—at least within a series of experiments. The study of lignocellulose breakdown, like that of lignin itself, has been stimulated by the ability to label the various components preferentially (Crawford and Crawford, 1976; Crawford, 1980) such that ^{14}C-[lignin]-lignocelluloses and ^{14}C-[glucan]-lignocel-

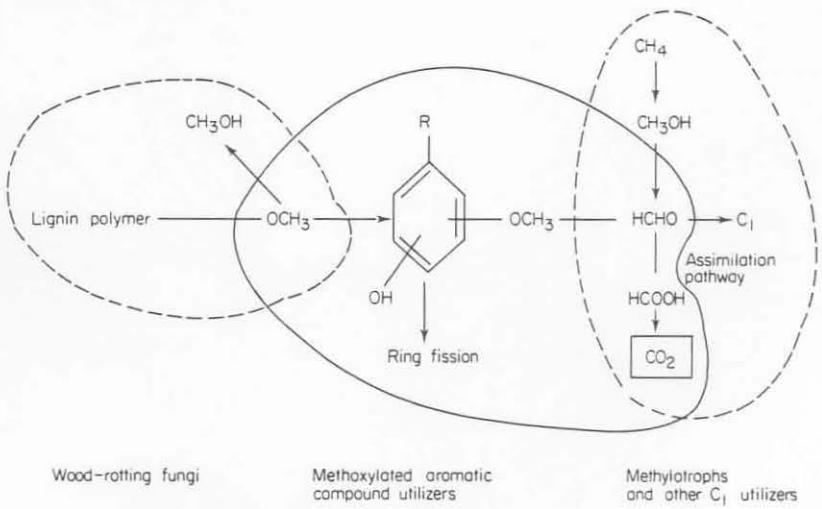

Fig. 2. Microbial communities implicated in lignin mineralization and their probable function (after Kuwahara, 1980).

luloses are now available. Consequently, it is possible to differentiate between those microorganisms merely degrading the glucan fraction, those degrading the lignin, and those degrading both. Using labelled lignocellulose Crawford and Crawford (1976) showed that *Thermonospora fusca* growing at 55°C was primarily utilizing the cellulose and not the lignin, whereas the white-rot fungus, *Polyporus versicolor*, degraded the entire substrate. Crawford and Sutherland (1979) discovered that whilst some *Streptomyces* species would decompose both principal components of lignocellulose and that others would only attack cellulose, none would solely degrade lignin. Phelan *et al.* (1979) examined a large number of actinomycetes and found six which decomposed lignin and cellulose simultaneously, although the former was more completely degraded than the latter. It is likely that some actinomycetes only remove side chains or methoxyl groups from lignin whilst other species are required in order to cleave aromatic rings (Trojanowski *et al.*, 1977; Phelan *et al.*, 1979). However, Crawford *et al.* (1980) have described a single *Nocardia* species which oxidises [^{14}C]-lignin completely to $^{14}CO_2$. Many of the studies of lignocellulose breakdown (Kirk *et al.*, 1976; Ander *et al.*, 1980; Hall *et al.*, 1980) suggest that microbial growth on the lignin component requires the presence of a co-substrate, such as cellulose, glucose, or even starch, so that whilst the presence of lignin is likely to depress cellulose hydrolysis, the presence of cellulose is essential for lignin decay to occur at all.

It appears that cellulases can be induced in the absence of close physical contact between the microorganism and its substrate (Rosenberg, 1979), presumably by a suitable diffusible molecule (Enari and Markkanen, 1977) or possibly through derepression in the absence of a diffusible molecule (Hulme and Stranks, 1971). In contrast, the production of lignin-degrading enzymes may require intimate contact (Drew and Kadam, 1979).

4.2.2 Ecological studies

Fungal species which effect the decay of polymeric wood tissue are broadly classified according to the nature of their substrates and the visual effect they have upon them. Accordingly, white-rot fungi extensively degrade all cell wall components, including lignin and cellulose, rendering the wood fibrous, spongy and bleached in appearance. Brown-rot fungi remove the cellulose and other polysaccharides but not the lignin leaving the wood in a cracked, brittle, and darkened condition. Soft-rots colonize the surface layers, rather than penetrating the wood, and degrade the cellulose, hemicellulose, and pectic components but generally not the lignin. They are particularly prevalent when the wood has a high water content but even then are regarded as having a limited degradative capacity when compared to the white and brown rots, especially when confronted with intact cell walls.

A fourth class of fungi, involved in plant material decay in general, is the sugar fungi (Garrett, 1963). Cells contain a variety of soluble sugars, amino acids, and uronic acids which form a suitable carbon source for the many fungi (and no doubt bacteria) unable to degrade the cell wall polymers. Those fungi utilizing the indigenous soluble carbohydrates present in undecayed plant material are sometimes referred to as primary sugar fungi; those growing on carbohydrates released by the depolymerizing activities of white, brown and soft rot fungi are known as secondary sugar fungi.

Swift (1977) differentiated between wood-inhabiting fungi (white- and brown-rots) and wood-invading fungi (soft-rots). Secondary sugar fungi were renamed secondary saprotrophs and their definition broadened to embrace fungi and bacteria (and arthropods) consuming the products of decay, microbial tissues, or even humus. A large and diverse group of microorganisms are concerned in mineralization processes at the secondary saprophyte stage once the plant material has been comminuted and degraded.

There have been few studies which have combined observations of microbial interactions during the degradation of carbon-containing organic debris with specific enzyme-substrate interactions. However,

there have been numerous investigations concerned with microbial colonization and succession in the breakdown of plant materials—some involving frequent sampling from plant debris where it is naturally deposited, some using enclosed and possibly defined substrates buried *in vivo* (e.g. litter bags), others involving decomposer communities *in vitro*. Thus we have a good deal of information as to the microorganisms involved in the mineralization of wood and leaves from a variety of species. A few representative studies are outlined here which, in addition to their ecological value, should be viewed as pointers towards community studies. In other words, likely combinations of bacteria and fungi could be selected for use in experiments designed to reveal the precise relationships of the organisms to each other and to a changing substrate.

Rayner (1978), in a study of fungal interactions in organic matter decay, observed the interactions *in vitro* between 26 different fungal species concerned in the degradation of hardwood. The experiments were conducted by inoculating malt extract agar plates with different pairs of fungi, one species on each side of the plate. Altogether some 200 combinations were tested and a range of responses recorded:

(i) formation of a pigmented zone at the point of contact;
(ii) development of a clear zone between the colonies;
(iii) production of leathery mycelium between the colonies,
(iv) lysis of one fungus by its pair;
(v) replacement of one fungus by its pair; and
(vi) stimulation of fruiting.

Species, such as *Hypholoma fasciculare*, *Phanerochaete vetulina*, *Phlebia merismoides* and *Scytalidium album*, were most effective at replacing other species whereas *Chondrostereum purpureum* was non-competitive and easily replaced by others. *Pha. vetulina* and *Phl. merismoides* lysed many of the opposing species before colonization.

With many of the combinations, opposing pairs of fungi were mutually antagonistic due either to the production of antibiotics (*Stereum hirsutum/Heterobasidion annosum*, *Pseudotrametes gibbosa/Coriolus versicolor*) or to the formation of a dense, leathery mycelial barrier (*Ganoderma adspersum/Daedaleopsis confragosa*). In an attempt to simulate a more natural environment Carruthers and Rayner (1979) paired various fungi on Petri dishes containing oak sawdust. *Phlebia merismoides* and *Hypholoma fasciculare* again proved to be amongst the most aggressive. A third level of study using inoculated wood stumps confirmed many of the results observed using malt extract agar.

Rayner and Todd (1979) summarized these and other studies and tentatively suggested a fungal succession sequence in the decay of

hardwood. Initially the virgin wood becomes colonized by a variety of parasitic and saprophytic microorganisms. The composition of this pioneer community will depend upon a number of factors including the part of the plant exposed (branch, bole, root), whether the wood is in contact with the soil, and the residual host resistance of the plant (occurrence of living cells, undamaged tissue). Aerially-exposed material tends to be colonized by soft-rot and stain fungi arising from spores; those portions on or below the ground are likely to be colonized by vegetative mycelium. Parasitic fungi commonly recorded during the early stages of decay include the familar basiodiomycetes *Fomes annosus* and *Armillaria mellea*. Several other species, which are not particularly active in decay and are subject to replacement by more aggressive fungi, are present at this stage: *Botrytis* spp., *Phialophora* spp., *Acremonium* spp., and the basidiomycetes *Chondrostereum purpurem*, *Shizophyllum commune*, *Crepidotus variabilis* and *Flammulina velutipes*.

In time, more active degraders expand to dominate the degradative community and these include the white rots *Coriolus versicolor*, *Stereum hirsutum* and *Hypholoma fasciculare*. Frequently these and other fungi present at this stage are mutually antagonistic and contact between them is restricted. The relatively undecayed interaction zones between antagonists may be colonized by species of *Cladosporium*, *Rhinocladiella*, *Endophragmiella* and *Catenularia* which may function as commensal secondary saprophytes dependant upon the products of their fungal neighbours (Rayner, 1976).

Eventually this somewhat stable phase is disrupted by other aggressive colonizers and a second replacement occurs. Fungal species involved in the second phase include *Phlebia merismoides*, *Phallus impudicus*, *Phanerochaete vetulina* and again *Hypholoma fasciculare*. As the wood becomes more extensively decayed the importance of these species may decline as the substrate is now suitable and accessible to a wide range of fungi (Mucorales, *Trichoderma* spp., *Scytalidium* spp.) and, of course, bacteria.

Plant litter decomposition was reviewed extensively by Dickinson and Pugh (1974) and recent advances have been discussed by Hayes (1979). For example, the fungal succession of aspen leaf litter in June and October was studied by Visser and Parkinson (1975). Rather than observe succession in progress, they isolated organisms from the litter, fermentation and humus layers of an aspen stand as representative of various stages of decomposition. As is to be expected, fallen leaves carry a microbial population with them which developed when the leaf was on the tree. A phylloplane flora consisting of 2×10^7 bacteria cm^{-2} and numerous fungi and yeasts was recorded by Ruinen (1961). In Visser

and Parkinson's (1975) study three principal saprophytes, *Aureobasidium pullulans*, *Pleurophomella spermatiospora* and *Cladosporium* spp., were present on net-caught leaves and of these *Cladosporium* spp. persisted for some while in the litter layer. Rapid replacement colonization occurred in the litter (L_1) and species of *Phoma*, *Discula*, *Mortierella*, *Phialophora*, and *Penicillium* were recorded. Many of these primary saprophytes are regarded as sugar fungi. Deeper litter layers (L_2), which represented the previous season's leaf fall, contained species of *Altenaria*, *Trichoderma*, *Mucor*, *Penicillium*, *Sclerotium*, and *Paecilomyces*. The authors suggested that these fungi were concerned either directly with cellulose decay or were associated with secondary saprotrophs (*Mucor*, *Mortierella*).

Most of the fungal genera in the L_2 layer persisted into the fermentation layer (F_1) but their frequency either increased (*Phoma*, *Trichoderma*) or decreased (*Discula*, *Paecilomyces*). Many of the fungi in the F_1 layer were assumed to be cellulolytic and lignolytic genera, whilst others (*Mucor*, *Absidia*, *Penicillium*) had a secondary saprotroph role. The same mineralization events were probably occurring in the F_2 layer, although some changes in microbial community structure were observed.

The final stage of succession in aspen leaf degradation was represented by the humus (H) layer. Many of the genera present in the F_2 layer also occurred in numbers here (excepting *Mucor*), although *Mortierella* and *Penicillium* increased in frequency whilst *Cylindrocarpon* appeared for the first time. It was proposed that the active depolymerization of leaf (cellulose, lignin) and fungal (chitin) constituents was taking place in the humus together with numerous secondary saprotrophic activities. Total fungal mycelium increased with depth. For instance in the October sampling the biomass (in g wet wt m^{-2}), in the various litter (L), fermentation (F) and humus (H) horizons, was L_1, 7.55; L_2, 16.99; F_1, 37.90; F_2, 40.15, and H, 188.34.

Frankland (19744, 1976) has reviewed the mycological and biochemical changes occurring during the decomposition of bracken litter (cellulose 37.5%, hemicelluloses 26%, lignin 28%, soluble carbohydrates 1.2%). She recorded approximately 390 species of fungi that were associated with senescent or dead *Pteridium aquilinum* petioles. Over a period of 5 to 6 years the changes in the community were monitored. Weak parasites, such as *Aureobasidium pullulans* and *Rhopographus pteridis*, were important primary colonizers of senescent and dead tissues and no doubt utilized the soluble cell carbohydrates. These species were replaced during the first year by primary saprophytes, such as *Cladosporium herbarum*, *Cylindrocarpon destructans*, and *Epicoccum*

nigrum, and Basidiomycetes (especially *Mycena galopus*) attacking lignin, cellulose, and hemicellulose. During the second and third years other Basidiomycetes accompany *Mycena* spp. in the succession along with a range of cellulolytic Fungi Imperfecti (*Trichoderma, Chloridium, Pestalotiopsis, Penicillium*). By the fourth year the fungal population was declining and was subject to predation by springtails and mites. However the nematode-trapping fungus *Dactylella megalospora* was becoming established and secondary sugar saprophytes (Mucorales, especially *Mucor hiemalis*) utilizing the products of cellulolysis were beginning to dominate the residual fungal community. The role of bacteria was more marked hereafter as the definable leaf litter merges into the soil organic matter.

Boois (1976) investigated fungal development on oak leaf litter and reported that *Aureobasidium pullulans*, as found by other workers using different substrates, was a dominant pioneer fungus in the decay of freshly fallen leaves. *Trichoderma*, *Penicillium*, and *Mortierella* species were common in the litter from 6 months onwards, *Mucor* species a little later.

Hering (1967) also looked at the fungal decomposition of oak leaves but chose a more classic approach: the incubation of previously sterilized leaves with individual fungal species. Of the ten fungal species tested the most active, in terms of loss of dry weight of oak litter at 3 and 6 months, were *Collybia peronata*, *Mycena galopus*, and *Cryptocline cinerescens*. *M. galopus* was particularly important as a degrader of cellulose, hemicelluloses and lignin. *C. peronata* was active against lignin and hemicelluloses but not cellulose, while *C. cinerescens* was ineffective against the lignin fraction. *Polyscytalum fecundissimum* was an abundant organism which decomposed cellulose and hemicellulose. Interestingly, although *Trichoderma viride* was common on old litter and is well-known as a cellulolytic fungus it had very little effect on the fresh leaves during the six month study. It seems that the cellulose in oak leaves, although theoretically a suitable substrate, is in someway unavailable to *Trichoderma*. Pioneer species, including the ubiquitous cellulose decomposers, *Aureobasidium pullulans* and *Cladosporium herbarum*, were present during the 6-month period but made a rather small contribution to weight loss.

Suberkropp and Klug (1976) investigated the microbial and chemical changes associated with the decay of leaves in an aquatic environment. Leaves of *Quercus alba* (white oak) and *Carya glabra* (pignut hickory) were incubated in a woodland stream and sampled biweekly for up to 32 weeks. Direct counts of bacteria increased exponentially with time although viable counts were generally lower. Bacterial

numbers were consistently lower on oak than on hickory but the composition was quite similar on both substrates. No obvious bacterial succession emerged but a range of genera were isolated from the leaves and identified: *Flavobacterium, Flexibacter, Pseudomonas, Acinetobacter, Achromobacter, Chromobacterium, Serratia, Alcaligenes, Bacillus, Cytophaga, Sporocytophaga*, and *Arthrobacter*. *Flavobacterium* and *Flexibacter* isolates were generally able to hydrolyse starch, pectin, casein, gelatin, and inulin, and only *Cytophaga* and *Sporocytophaga* used cellulose and cellobiose as carbon sources. The authors suggested that most of the bacteria may obtain their carbon from fungal metabolites, lysed fungal cells, or the products of proteolysis as they were unable to degrade the structural cell wall carbohydrates. Bacterial biomass increased in the later stages of decomposition and was paralleled by a decline in the fungal population.

Suberkropp and Klugg (1976) further showed that aquatic phycomycetes (e.g. *Alatospora acuminata, Flagellospora curvula, Tetracladium marchalianum*) were the dominant mycoflora on both leaf species although abundance and patterns of succession differed. *F. curvula* was dominant during the first 4 to 6 weeks on both oak and hickory and declined rapidly thereafter. *Lemonniera aquatica* was also an early colonizer but maintained its presence throughout the experimental period. With the decline of *Flagellospora curvula*, *Alatospora acuminata* become the dominant species on oak and the co-dominant species (with *Tetracladium marchalianum*) on hickory. A large number of soil fungi were also present (e.g. *Alternaria, Penicillium, Fusarium, Aspergillus, Cladosporium*) but in the dormant state, only growing when plated onto rich media. All the aquatic hyphomycetes were capable of producing extracellular pectinase and cellulase.

The differences in microbial colonization between the two leaf species was considered to be due to inherit differences in their chemical composition as well as changes occurring during decay. For instance, soluble polyphenolics and lignins are more abundant in oak than hickory (28% compared with 19%). It is well known that phenolics complex with proteins (Benoit and Starkey, 1968) and therefore these aromatics may be inactivating the exoenzymes and thus reducing fungal growth as well as that of any bacteria which depend upon fungal metabolites. Changes in the dominant microflora during substrate breakdown are no doubt influenced by changes in soluble carbohydrates (largely disappeared during the first two weeks), cellulose (gradual decline after 2 to 4 weeks) and hemicellulose (gradual decline after 12 weeks—oak, and after 2 weeks—hickory).

Goodfellow and Dawson (1978) chose to investigate bacterial popu-

lations in the L, F, and H layers of a highly acid spruce forest soil over a period of one year. The highest numbers of bacteria were recorded in the humus layer although actinomycetes and microfungi did not follow this pattern. There was a dramatic drop in bacterial counts in the top layer (A horizon) of the underlying mineral soil. The commonest, positively identified bacteria in the L layer were *Arthrobacter* spp. and *Bacillus firmus*. The occurrence of these species declined sharply in the F layer where other bacilli (*B. lentus*, B. polymyxa, *B. coagulans*, *B. sphaericus*) and *Micrococcus* spp. were dominant. In the H layer yet other bacilli became important (*B. chitinosporus*, *B. cereus*, *B. globisporus*, *B. laterosporus*). The absence of *Achromobacter, Pseudomonas*, and *Flavobacterium* species from the litter horizons is somewhat surprising as these are generally considered to be amongst the commonest litter-degrading bacteria (Jensen, 1974), but there is no doubt than Gram-positive organisms were in abundance in this soil. Although the streptomycetes were not described other than by colony characteristics, a similar pattern was seen with certain strains being dominant in one layer yet missing from another.

It is never easy to explain these observations without further information regarding the enzymic capacity of the microflora, the changing morphology and biochemistry of the leaf litter, and the physicochemical properties of the various soil horizons. Appropriately, a recent study adopted an enzymological approach to leaf decay. Spaulding (1977) measured the activities of a number of enzymes extracted from coniferous leaf litter including cellulase, xylanase, peroxidase, amylase, and invertase. This investigation revealed a definite correlation between carbon dioxide production and amylase, cellulase, and xylanase activities although amylase may be involved in microbial cell turnover rather than the mineralization of litter which contains little starch. Enzymic determinations such as these, together with observing substrate destruction *in situ* with the electron microscope should enable us to construct a more accurate picture of leaf carbon mineralization. An example of the latter is provided by Kilbertus and Reisseнger (1975) who incubated plant debris both on and within soil and then used transmission electron microscopy to examine thin plant sections for microbial species and associated areas of enzymic erosion. They were able to observe the cellulolysis caused by *Cladosporium herbarum*, *Stachybotrys chartarum*, and a *Chaetomium* sp., and the destruction of starch by *Mortierella ramanniana*. After 60 days there were signs of pectolytic, lipolytic and lignolytic activities. The progress of wood colonization and decay by actinomycetes has also been followed using scanning electron microscopy (Baecker and King, 1980).

A different approach to understanding the enzymic basis of carbohydrate decay is illustrated by a recent study to assess the likely involvement of various *Aspergillus* species in the deterioration of stored grain (Flannigan and Bana, 1980). These workers tested a range of aspergilli for amylase, α- and β-glucosidase, β-(1-3)-glucanase, pectinase, lipase, xylanase, CMCase, and other enzymes. On the basis of their enzyme activities certain species (e.g. *Aspergillus glaucus* group) were regarded as less likely to cause extensive degradation of grain than were other species (*A. flavus, A. terreus*). This work illustrates a separate source of information concerning the contribution of microorganisms to carbon mineralization, namely that derived from biodeterioration research (Oxley *et al.*, 1980).

Just a few of the many studies of microbial succession during the decay of plant materials have been mentioned here. They suffice to indicate the plethora of data which can be used to design experiments which may reveal the interactive nature of microbial species in the mineralization of complex organic substrates.

Whilst this chapter is primarily concerned with the direct action of microorganisms on complex botanical substrates it should not be forgotten that, in general, microfauna (nematodes, protozoa, rotifers), mesofauna (collembola, diptera, larvae, termites) and macrofauna (millipedes, insects, molluscs, earthworms) all have important comminutive and catabolic roles in the degradative community. More specialized contributions are made by wood-boring insects which stimulate breakdown by forming channels in the substrate, thus encouraging aeration and exposing more of the material to microbial colonization. Insects also serve as vectors for microbial inoculum. In addition some insects harbour microorganisms in their gut which are capable of depolymerizing cellulose, other polysaccharides, and possibly even lignin (Seifert and Becker, 1965). Wood digestion by termites has been reviewed recently by Stradling (1977), and Grosovsky and Margulis (1982). Some termite species cultivate (and subsequently graze upon) cellulolytic and lignolytic fungi on the walls of their burrows. For a detailed description of the whole spectrum of decomposer organisms the reader is referred to Swift *et al.* (1979).

4.2.3 Biotechnological studies

Many of the fermentation studies concerned with complex substrates have utilized two or more microbial species. Srinivasan and Han (1969) investigated the breakdown of sugar cane residues (bagasse) by a *Cellulomonas* sp. and discovered that protein production (i.e. yield of microbial biomass) was increased if an *Alcaligenes* species was included

in the fermenter. In this two-membered community the *Alcaligenes* consumed the cellobiose which would otherwise have inhibited cellulose production by the *Cellulomonas* species. *Candida guillermondi* and *Trichosporon cutaneum* have been used to remove cellobiose produced by *Cellulomonas* (Srinivasan, 1975). A different relationship was shown by Ghose *et al.* (1976) who used the same form of crude cellulose (46% cellulose, 25% hemicellulose, 21% lignin) but looked at the effect of *Trichoderma viride* and *Aspergillus wentii* culture filtrates on sugar yield. A 1:1 mixture of the enzymes increased the rate of reducing sugar production when compared to *T. viride* enzymes alone. Apparently, this relationship depends upon the contribution of *Aspergillus wentii* to xylan degradation and presumably the resulting increase in the accessibility of cellulose to the *T. viride* cellulases. The mixed population had no such stimulatory effect upon cellulose alone.

Wheat straw (40% cellulose, 29% hemicellulose, 14% lignin) has been chosen as a realistic cellulosic substrate by a number of workers. The lignin component in straw is bound tightly to the cellulose and hemicellulose fractions, effectively reducing their accessibility to degradative enzymes (Cowling and Brown, 1979; Kirk, 1975). Thus, in a great many experiments, designed to study protein production, physical or chemical pretreatment, is used prior to inoculation. Chahal *et al.* (1979) subjected wheat straw to sodium chlorite to remove the lignin component and found that all microorganisms tested were more effective at degrading the modified holocellulose (cellulose + hemicellulose) substrate. The microorganisms, which included *Aspergillus niger*, *Aspergillus terreus*, *Cochliobolus specifer*, *Myrothecium verrucaria*, *Rhizoctonia solani*, *Spicana fusispora*, *Penicillium* sp., and *Gliocladium* sp., were all originally isolated from decomposing wheat straw. *Cochloibolus specifer* was the most efficient (in terms of protein production) of all species with all substrates: untreated straw, lignin-free straw (holocellulose) and cellulose alone. Hemicelluloses may also slow down the rate of fungal degradation of straw due to the presence of somewhat resistent sugars arabinose and galactose, and methylation (Moo-Young *et al.*, 1978). Other axenic culture studies of wheat straw decay have been carried out by Moo-Young and co-workers (Chahal *et al.*, 1977; Moo-Young *et al.*, 1978, 1979) especially with *Chaetomium cellulolyticum* which they say compares favourably with other fungi in terms of biomass production. No doubt one of the main reasons for this efficiency is that *C. cellulolyticum* rapidly utilizes cellobiose.

Peitersen (1975) used a mixed culture of *Trichoderma viride* and a yeast (*Candida utilis* or *Saccharomyces cerevisiae*) for the breakdown of pretreated straw. The yeast was inoculated some 24 to 32 h after the fungus. In

comparison to *T. viride* alone, the time taken for maximum yield of cellulases and cell protein was reduced by several days. This was assumed to be due to the removal of glucose by the yeast which otherwise repressed *Trichoderma* cellulase production. An additional nutritional benefit may arise from lysed yeast cells. Communities of microorganisms involved with other fermentations of industrial importance are fully described elsewhere in this volume (Drozd and Linton).

4.3 Microbial Debris

Despite the accepted importance of microbial residues as substrates for microbial growth, there have been few attempts to describe the communal and successional events concerned in their mineralization. Furthermore, only occasionally are the actual decomposer organisms named, with research efforts having been concentrated on overall mineralization rates. Many recent mixed culture studies have also acknowledged the importance of the products of cell lysis to one or more members of the community but rarely is the nutritional basis of the relationship explained.

Research prior to 1970, particularly as it refers to the mineralization of microbial debris in soil, is reviewed by Webley and Jones (1971). Not surprisingly, chemically-different cellular constituents (lipopolysaccharides, chitin) and therefore cell components (cell wall, cytoplasm) are subject to different rates of mineralization. Generally cytoplasmic fractions are degraded more rapidly than cell wall fractions (Verma and Martin, 1976) whilst according to Nakas and Klein (1980) bacterial cell walls were more susceptible to decomposition than their fungal counterparts, the reverse being true for the cytoplasmic components. It has been known for some while that cell wall pigments, such as melanin, will retard mineralization (Martin *et al.*, 1959; Kuo and Alexander, 1967). Recently Linhares and Martin (1978) measured the $^{14}CO_2$ evolution from the breakdown of labelled fungi incorporated in soil. Over a 12-week period considerably less carbon dioxide was released from melanic fungi (*Hendersonula toruloidea*, *Aspergillus glaucus*, *Eurotium echinulatum*) than from hyaline species (*Penicillium vinaceum*, *Penicillium nigricans*). The rate of carbon mineralization was also reduced by complexing cells and cell components with various phenolics (Verma and Martin, 1976; Nelson *et al.*, 1979), a process that is known to occur naturally as the products of microbial decay become associated with humic matter (Mayaudon and Simonart, 1963; Mayaudon, 1966). Jones and Webley (1968) approached the study of microbial cell degra-

dation somewhat differently. These workers incorporated fungal wall material (from *Fusarium culmorum* and *Mucor ramannianus*) into soil aggregates and monitored colonization and monitored colonization and β-(1-3)-glucanase activity. There was a good relationship between the occurrence of *Streptomyces* spp., the degradation of *Fusarium* and β-(1-3)-glucanase activity.

5. CARBON MINERALIZATION IN THE SOIL ENVIRONMENT

5.1 Introduction

Approximately 10% of the organic matter in soil occurs as carbohydrate and most of that is as polysaccharide. In mineral and cultivated soils carbohydrates account for less than 2% of the soil weight, whereas in organic soils (e.g. peat) values may be as high as 30%. The degradation products of plants, insects, mammals, and microorganisms all contribute to the carbon pool and, in addition, some novel polysaccharides and polyphenolics are synthesized. Soil polysaccharides of microbial origin contain hexoses and deoxyhexoses; those containing arabinose and some of those containing xylose are believed to be derived from plants (Cheshire, 1977).

Microorganisms degrade carbon-containing substrates in soil as in other environments in order to build new cells. In biogeochemistry this process is termed immobilization, as it removes carbon from the immediate attentions of the decomposer community. However, with the death of the first flush of degraders the microbial cells are mineralized and a further proportion of the original carbon is released as carbon dioxide. In other words, each generation of microorganisms is actively involved in immobilization and mineralization yet subsequently serves as substrate for the next generation. Microbial tissues may even be more accessible to decomposers than plant material (Kaszubiak *et al.*, 1976) and the proportion of the original substrate carbon that is mineralized compared to that which is immobilized in new cell material varies with microbial species, substrate, availability of other essential elements (i.e. carbon:nitrogen; carbon:phosphorus; carbon:sulphur ratios) and the physico-chemical nature of the environment (e.g. pH, redox potential, adsorptive properties). The aerobic degradation of substrates in soil may be reasonably efficient. Jenkinson (1968) and Shields *et al.* (1973) estimated that during the primary decomposing cycle, the microflora converts between 40 and 60% of the

original carbon to microbial tissues. Anaerobes, by comparison, may assimilate less than 5% of their carbon supply (Wagner, 1975). Fungi and bacteria are believed to make equal contributions to soil respiration (Clarholm and Rosswall, 1980). Assuming that, at least in an aerobic environment, equal proportions of carbon are immobilized and mineralized, then primary decomposition releases 50% of the carbon dioxide, whilst successive waves of secondary decomposers utilizing microbial cell carbon will release 25% of the residual carbon (75% total), 12.5% (87.5%) and so on. Theoretically after six generations (one primary, five secondary) 98.5% of the initial carbon source will be mineralized (i.,e. evolved as carbon dioxide). However, this is an oversimplified view of mineralization because at least one other pool of organic carbon is formed during substrate decay—humic matter.

The humic fraction of soils and sediment contains a variety of chemically and physically associated carbonaceous substrates including amino acids and proteins, incompletely degraded carbohydrates and lignin components, as well as novel polyphenolics synthesized during microbial proliferation. The heterogeneity of humic materials means that its components display different susceptibilities to microbial attack or to abiological release and therefore have different residence times in soil. A frequent estimate is that between 2 and 5% of humus carbon is mineralized each year (Alexander, 1977), although additions of easily degradable substrate, such as green manure, may accelerate carbon dioxide release—a process known as "priming" (Jenkinson, 1966). The most recalcitrant aromatic polymeric components of humic colloids may have half-lives measured in hundreds of years (Campbell *et al.*, 1967; Sorensen, 1975). Thus, in a temporal sense, there is a third phase of mineralization; the gradual conversion of humic carbon to carbon dioxide. Many attempts have been made to assess residence times of various forms of organic carbon in soil (Wood, 1974; Lousier and Parkinson, 1976; Paul and Voroney, 1980) and the influence of clay and organic colloids upon substrate persistence (Cheshire, 1979). Primary, secondary, and tertiary processes in mineralization are depicted in Fig. 3.

5.2 Factors Affecting Persistence of Carbon-containing Substrates in Soil

It appears that certain soil constituents have a protective effect upon polysaccharides (Cheshire, 1977) and it has been shown that purified enzymes have a reduced degradative capacity when presented with polysaccharides from extracted soil (Cheshire and Anderson, 1975).

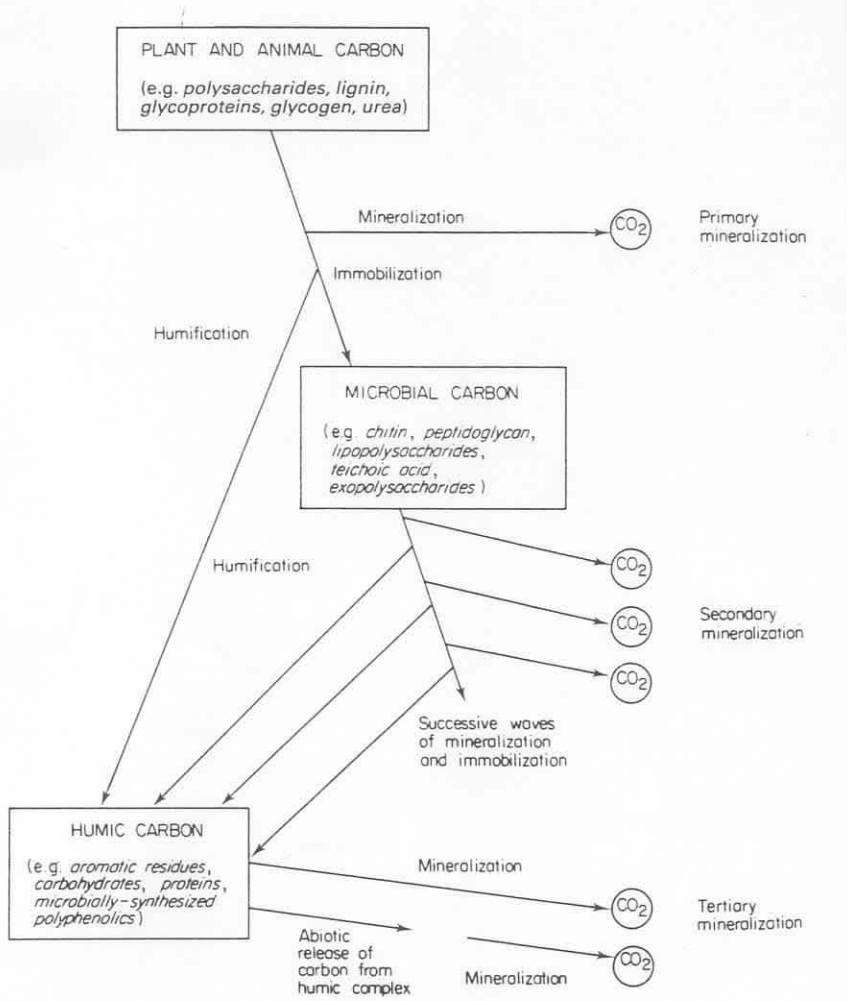

Fig. 3. Levels of carbon mineralization.

Without doubt any differences in stability between polysaccharides observed *in vitro* can be related to intrinsic factors such as sugar composition, the nature of substituents and the presence of other cell components. *In vivo*, however, the soil inorganic and abiotic organic components are believed to contribute significantly in three different ways to the unexpected recalcitrance of polysaccharides in soil.

Firstly, polysaccharides are known to be adsorbed to a certain extent by clays (Parfitt and Greenland, 1970). Polysaccharides can enter the

interlamellar spaces of expanding-lattice clays (Greenland, 1956; Finch *et al.*, 1967), a location which will protect the carbohydrate from the direct attention of microorganisms and which may even hinder the approach of exoenzymes. Incidentally, chemical degradation may also be slowed down due to adsorption. The adsorption of a polysaccharide in excess of that predicted by its molecular size and the surface area of the clay adsorbant (Olness and Clapp, 1973, 1975) may occur if only part of the carbohydrate polymer is attached (Hayes and Swift, 1978) or if a multilayer is formed. Various adsorptive mechanisms have been proposed to account for the attachment of polysaccharides to clays and these include: hydrogen bonding (Kohl and Taylor, 1961; Finch *et al.*, 1967); complex formation (Parfitt, 1972); and metal ion bridging (Saini and MacLean, 1966; Guckert *et al.*, 1975). Parfitt and Greenland (1970), however, believe that multiple dispersion forces and entropy changes, arising from the replacement of small molecules, such as water, at the clay surfaces, are sufficient to account for the adsorption of polysaccharides.

Secondly, many polysaccharides form complexes with metal ions, such as calcium, zinc, iron, and aluminium, which will reduce their rate of degradation (Martin *et al.*, 1966, 1972).

Finally, the modifying effect of phenolic compounds on polysaccharide degradation has been recognized for many years (Handley, 1954; Benoit and Starkey, 1968). Phenolics may occur either as a component of plant material which is released during the decay of lignin-containing organic matter or be synthesized *de novo* by the microflora. Either way they may protect polysaccharides in a physical sense (Swincer *et al.*, 1969) or by forming a chemical association (Martin *et al.*, 1978). Phenolics may also protect substrates by specifically inhibiting the microorganisms which would otherwise degrade them (Ivarson, 1977). The persistence of polysaccharides concerned in soil aggregate formation may be due to their subsequent tanning (Griffiths and Burns, 1972).

5.3 Extracellular Carbohydrases in Soil

Soil and sediment-dwelling microbial communities, which depend upon exogenous carbon sources for their nutrition, are confronted with enormous problems. For instance, suitable substrates are discontinuous in both time and space, and there may be prolonged periods during which the microbial cell must rely upon maintenance factors, particularly if it is unable to find refuge in endospore formation. Those micro-

organisms that utilize polysaccharides have even greater problems in that they need to excrete suitable enzymes whose chance of success is remote. In other words, extracellular polysaccharases are released into an environment in which adsorption, denaturation and degradation take a rapid toll of diffusible enzymes. Any enzyme surviving these obstacles must then encounter a suitable substrate, react with it (if the pH is favourable) and release a lower molecular weight product with increased solubility. The product must subsequently return to the microorganism which secreted the enzyme in the first place, in order that the energetic deficit caused by the production and secretion of extracellular enzymes should be made up and that the microbe should gain a nutritional advantage from its efforts. However, the product is now a suitable substrate for a large number of species in addition to the specialist microorganism which produced the depolymerase, or it may become attracted to and immobilized on a particulate surface (e.g. clay). Whatever, both factors serve to reduce the amount of substrate reaching the enzyme-producing microorganisms.

There is some evidence to support the notion that the soil is a hostile environment for free enzymes. This derives from two sources: the first is that enzymes added to soil (or stimulated within it) are rapidly inactivated (Drozdowicz, 1971; Zantua and Bremner, 1976); the second is that enzymes have great difficulty in diffusing through soil barriers to reach their substrates (R. G. Burns and C. F. A. Hope, unpublished observations).

Because of these constraints it is difficult to envisage a successful strategy for substrate utilization and microbial growth in soil founded upon the production of diffusible exoenzymes. Certainly the continual release of enzymes from microorganisms, whilst profitable in a flask containing abundant substrate, seems doomed to failure in the soil environment. Now we have already seen that in some but not all cellulolytic bacteria the diffusion of extracellular enzymes is curtailed by their retention at the cell wall. This approach will certainly improve the survival and efficiency of the cellulase, even though the enzyme cannot at the same time be involved in scavenging for substrate. Nonetheless, direct contact between cell wall-associated enzyme and substrate must improve the microorganism's chance of success in that the soluble products of any catalysis are available for immediate uptake, and are not subject to attenuation by physical, chemical, and biological forces. This type of intimate interaction does not, however, explain how the microbe detects and locates the substrate in the first instance. A chemotactic response must be initiated by a soluble attractant, and yet we have already stated that it is unlikely that

microbial exoenzymes survive in the soil aqueous phase and even if they did the likelihood of a soluble product reaching the producing cell (and stimulating a tactic response) is remote. Therefore, when considering the microbial mineralization of carbon-containing substrates *in vivo*, it may be necessary to search for a more satisfactory strategy—possibly involving "accumulated" hydrolases.

Accumulated enzymes form an extracellular catalytic-component of soils and sediments that is stabilized through its association with the colloidal organic (humic) and inorganic (clay) constituents. These enzymes have a longevity not normally characteristic of free enzymes. The origins of accumulated enzymes and the nature of their association with the soil colloids has been debated exhaustively (Skujins, 1976; Burns, 1977, 1978) and a current view is that these enzymes are derived predominantly from microorganisms and are co-polymerized with humic matter during its formation. The resulting complex affords the enzyme some protection whilst still allowing it to retain a proportion of its activity (Rowell *et al.*, 1973; R. G. Burns and J. P. Martin, unpublished observations). In some instances the clay colloid, as such, may have a protective effect on enzymes (Stotzky, 1972) although it is more common for the clays to protect enzymes indirectly in the sense that they are stabilizing the organic matter. Accumulated hydrolase levels are at a steady-state condition in mature, climax soils. In other words, any leakage and destruction of enzyme is counterbalanced by the continuing process of humification during which enzyme is incorporated into novel polyphenolics. A large number of carbon substrate hydrolases have been reported as existing as colloid-bound moieties in soil (Ladd, 1978—Table 3) and, even allowing for the sometimes less than adequate methods of detection employed, it is apparent that humic matter has a significant enzymic capacity associated with it. By far the most frequently described hydrolase is urease (EC 3.5.1.5) which is suggested as responsible for a high proportion of the rapid urea hydrolysis in soil (Paulson and Kurtz, 1969; Pettit *et al.*, 1976). This ubiquitous accumulated enzyme illustrates an important characteristic of soil enzymes: they are not derived solely from enzymes whose normal functional location is extracellular. Urease, of course, is a cytoplasmic enzyme depending upon a soluble, low molecular weight substrate. Thus it may be inferred that a proportion of enzymes arising from lysed cells and those leaking from live cells can be incorporated into humic polymers as well as extracellular enzymes *sensu stricto*. Other accumulated enzymes involved in carbon mineralization include amylases, a variety of cellulases, β-D-glucosidase, β-1,3-glucanase, xylanase, and pectinase. A comprehensive list is shown in Table 3.

Table 3. Accumulated carbohydrases (Ladd, 1978).

Recommended Name of Enzyme	EC Number
α-amylase	3.2.1.1
β-amylase	3.2.1.2
cellulase	3.2.1.4
endo-1,3(4)-β-D-glucanase	3.2.1.6
inulinase	3.2.1.7
endo-1,4-β-D-xylanase	3.2.1.8
dextranase	3.2.1.11
polygalacturonase	3.2.1.15
α-D-glucosidase	3.2.1.20
β-D-glucosidase	3.2.1.21
α-D-galactosidase	3.2.1.22
β-D-galactosidase	3.2.1.23
levanase	3.2.1.65

Are accumulated enzymes important entities in soil or do they merely make a fortuitous and trivial contribution to microbe–substrate interactions? Unfortunately, there is no unequivocal answer to this question although an ecological role for accumulated enzymes has been proposed (Burns, 1979; 1980). This model suggests that the humic-enzyme complex serves as a stable detector of exogenous substrates which passes on the information (in the form of soluble inducer or derepressor molecules) to adjacent microorganisms. If the substrate concentration is high enough then the signal received will cause the microorganisms to assume the major role in substrate decay. Thus communal activities *in vivo* should perhaps be envisaged as involving both cells and immobilized exoenzymes juxtaposed and interacting at the soil/liquid interface.

6. CONCLUSIONS

It would be naive to view the mineralization of any carbon-containing polymer as the function a single microbial species. Even though a few microbial species in axenic culture are capable of producing the array of enzymes necessary to convert pure forms of cellulose, starch, or xylan to carbon dioxide *in vitro* it is unlikely that these organisms will perform the same function independently when confronted with the substrate *in situ*. This is due to four principal differences: an increase in the physical and chemical complexity of the native substrate (e.g. pure cellulose

fibres compared to those imbedded in and associated with lignins, pectins, and hemicelluloses); the intense inter-species rivalry inevitable in a largely oligotrophic habitat; an environment where the temperature, pH, and level of hydration and aeration may be a long way from the optimum conditions provided *in vitro*; and the extensive charged, and therefore reactive, surface areas provided by colloidal organic matter and clays. In order to overcome these constraints it is apparent that microorganisms have surrendered their independance and instead formed mixed species communities which co-operate in the breakdown of natural and synthetic substrates.

It is thus helpful to think of the mineralization of organic matter in terms of microbial communities and indeed a holistic view of soil as a complex multicellular body collectively responding to substrates is not new (Quastel, 1965; McLaren and Peterson, 1967). Furthermore the function of persistent colloid-bound enzymes, proposed on p. 522, implies that the overall microbial community has spatial and temporal dimensions reaching beyond those delineated by proliferating microorganisms. Recently the editors of *Contemporary Microbial Ecology* (Ellwood *et al.*, 1980) wrote that it was useful "to consider the total gene pool of a given mixed microflora located in a particular environment in determining the response of individuals or interacting groups of organisms to . . . whatever influences the growth of microorganisms or communities". The implications of this statement extend even beyond the cellular co-operation involved in mineralization to include the exchange of metabolic capabilities through bacterial conjugation (Slater and Godwin, 1980).

Microbial ecologists acknowledge that the remarkable capacity of terrestrial and aquatic environments for dealing with an enormous volume and range of substrates is due, at least in part, to the co-operative nature of microbial communities. Further to this it is usually accepted that microbial communities have a degree of flexibility and adaptability when placed under stress during natural and man-made perturbations. Some of this resilience may be due to the facultative nature of microbial communities, not in the sense that its members do not require a communal existence for success but rather that a variety of different microbial combinations are capable of the mineralization of a particular substrate and that their relationship to each other is casual in terms of the particular species involved. In other words, if one component of a community is inhibited another will take its place.

Studies of microbial communities and carbon mineralization have tended to concentrate upon the colonization and succession aspects of macro-organic matter decay. Unfortunately, these ecologically-

oriented studies have rarely revealed the nutritional relationships between microorganisms and their substrates nor have they emphasized the communal aspects of the various microbial species involved. Nonetheless Rayner and Todd (1979) are quite unambiguous about the importance of interacting microbial communities in organic matter decay, and have warned against considering the process as performed by broad waves of microorganisms influenced solely by physical and nutritional changes in the substrate. Indeed they say that "rather than ask what is succession (in organic matter breakdown) a more pertinent question is what is the fungal community, how is it maintained and what forces change it?"

More recently our understanding of the nature of microbial communities has been advanced by an ever-increasing number of mixed culture studies. These investigations have been carried out largely in continuous culture although the relevance of this environment to a soil, sediment or aquatic situation, where substrates are in low concentrations and are unevenly dispersed (Duursma, 1961; Gray, 1976), is questionable. Nonetheless, the concept of biogeochemical cycles implies the mobility of continuous culture rather than the closed environment of the batch culture; in reality, natural environments may fall somewhere between the two: chemostats subject to irregular pulses of substrate. Major considerations in the design of chemostat studies for monitoring community degradation of polysaccharides include:

(i) the changing chemical and physical nature of the substrate during mineralization;
(ii) the difficulty of including antagonistic microorganisms, such as protozoa, which may be important factors in maintaining a favourable nutritional balance between the primary organisms concerned in polysaccharide decay and therefore the stability of the community (Fenchel and Jørgensen, 1977);
(iii) the feasibility of introducing an insoluble, particulate carbon source;
(iv) the use of growth-limiting concentrations of substrate which may select a different (and more realistic) microbial community with low saturation constants and low maximum specific growth rates (Matin and Veldkamp, 1978); and
(v) whether soil or its components should be included in order to provide extensive anionic surface areas as well as a physical refuge for the less competitive and yet important members of the community.

An isolated attempt to simulate a complex soil/microorganism/macro-organic matter community in a fermenter vessel has been

described by Lynch and Gunn (1978). These workers monitored the physico-chemical changes occurring during wheat straw breakdown in soil slurries but did not describe the microflora concerned.

Another approach to understanding carbon mineralization which has attracted some attention involves the use of mathematical models (Bunnell and Scoullar, 1975; Smith, 1979a,b; Swift *et al.*, 1979) to describe the transit of carbon through biological cycles. As with all models these descriptions are only useful if they allow the meaningful interpretation of existing data, stimulate the production of new data, and are flexible enough to embrace unforeseen results.

It is quite obvious from this review that our knowledge of microbial communities involved in naturally-occurring substrate mineralization is paltry. It is not difficult to find reasons for this when one considers the physical and chemical diversity of the substrates and the properties of the terrestrial and aquatic environments where degradation occurs. These factors have conspired with conventional pure culture microbiology to persuade the research worker to study defined and homogeneous substrates in axenic culture. Thus I make no apology for presenting an extensive list of substrates and a detailed description of the mineralization of the commonest carbon polymer, cellulose. This is where the current emphasis lies. However, this information should now form the basis of a gradual return to the study of communities composed of two or more microbial species degrading heterogenous substrates. There are a great many guide-lines deriving from existing investigations of the microbial succession and colonization of organic debris as well as from studies of the rumen and of fermentation processes in general. In addition, the expanding interest of microbial physiologists in mixed cultures and their mathematical description is an important stimulus. McFadyen (1975), when writing about ecology in general, warned of the dangers of a totally descriptive approach, "a bottomless pit of precious time, effort and enthusiasm". Perhaps, as the appearance of this volume implies, we are now ready to pass from the descriptive phase of the role of microbial communities, to an analytical phase, and thence to a synthetic and even predictive phase.

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